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Advanced technologies to obtain functional ingredients from shiitake
mushrooms

(Lentinula edodes)

Tecnologías avanzadas para la obtención de ingredientes funcionales de shiitake

(Lentinula edodes)

Memoria presentada por

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Que el trabajo recogido en este documento titulado “Advanced technologies to obtain functional ingredients from shiitake mushrooms (*Lentinula edodes*) / Tecnologías avanzadas para la obtención de ingredientes funcionales de shiitake (*Lentinula edodes*)”, y que constituye la memoria presentada por D. Diego Morales Hernández para optar al grado de Doctor en Ciencias de la Alimentación, ha sido realizado bajo su dirección en el Instituto de Investigación en Ciencias de la Alimentación (CIAL) y la Universidad Autónoma de Madrid.

Y para que así conste, firman el presente informe en Madrid, a 9 de septiembre de 2019.

Fdo.: Dña. Cristina Soler Rivas.

Fdo.: D. Alejandro Ruiz Rodríguez

A mis padres.

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Abbreviations

ACE: Angiotensin converting enzyme	HSQC: Heteronuclear Single Quantum Correlation
ALT: Alanin aminotransferase	HWE: Hot water extraction
ASE: Accelerated solvent extraction	LDL: Low density lipoprotein
AST: Aspartate aminotransferase	LPS: Lipopolysaccharide
ATCC: American Type Culture Collection	MAE: Microwave-assisted extraction
BGE: β -D-glucan-enriched extract	MERG: Microemulsified ergosterol
BMI: Body mass index	MF: Microfiltration
CVD: Cardiovascular disease	MP: Mushroom powder
DB: Degree of branching	MS: Mass spectrometry
DEPT: Distortionless enhancement by polarization transfer	MSFE: Microemulsified supercritical fluid extract
DMM: Dietary mixed micelle	MW: Molecular weight
EFSA: European Food Safety Authority	NF: Nanofiltration
ERG: Ergosterol	NMR: Nuclear magnetic resonance
FDA: Food and Drug Administration	PEF: Polysaccharide-enriched fraction
FID: Flame ionization detector	PeF: Pulsed electric field
FIP: Fungal immunomodulatory protein	PLE: Pressurized liquid extraction
FOSHU: Food for Specific Health Use	PSC: Polysaccharides
FTIR: Fourier transform infrared spectroscopy	PWE: Pressurized water extraction
GALT: Gut-associated lymphoid tissue	qPCR: Quantitative polymerase chain reaction
GC: Gas chromatography	RDA: Redundance analysis
GRAS: Generally Recognized as Safe	RF: Retained fraction
HDL: High density lipoprotein	RIP: Ribosome inactivating protein
HFHD: High fat hypercholesterolemic diet	RO: Reverse osmosis
HMGCR: 3-Hydroxy-3-methylglutaryl-CoA reductase	SAHH: S-adenosyl-L-homocysteine hydrolase
HPLC: High performance liquid chromatography	SEE: Subcritical ethanol extraction
HPSEC: High pressure size exclusion chromatography	SFC: Supercritical fluid chromatography

SFE: Supercritical fluid extraction

SPE: Steam pressurized extraction

SWE: Subcritical water extraction

TSS: Total soluble substances

TSP: Total suspended particles

UAE: Ultrasound assisted extraction

UV: Ultraviolet

WC: Waist circumference

WIPO: World Intellectual Property Organization

WS: Wide spectrum

Summary/Resumen

Summary

Nowadays, consumption of edible mushrooms is encouraged by nutritionists and health-caring authorities, not only because of their culinary and nutritive value but also because they contain bioactive molecules that can be beneficial for human health. Shiitake mushrooms (*Lentinula edodes*) deserve special attention since they showed a wide diversity of health-promoting activities such as hypocholesterolemic, immunomodulatory, antioxidant, antitumoral, antihypertensive, etc. These biological properties were related with specific compounds e.g. fungal sterols (ergosterol and derivatives), polysaccharides (α - and β -D-glucans, chitins, etc.), proteins/peptides and secondary metabolites (phenolic compounds, eritadenine, lenthionine, etc.). Therefore, the aim of this PhD thesis was to evaluate the use of several advanced extraction technologies that can be up-scaled and sequentially applied or combined to obtain functional ingredients from shiitake mushrooms to design novel functional foods.

Supercritical CO₂ (SFE) procedures at pilot and larger scales were adjusted to obtain fungal sterols-enriched extracts. These extracts could be transformed into ergocalciferol-enriched fractions (vitamin D₂) by submitting them to UV-irradiation. The supercritical extracts contained up to 53% ergosterol and they were able to displace cholesterol from dietary mixed micelles using an *in vitro* digestion model. They were also effective when the extracts were microemulsified and mixed with fungal β -D-glucans. However, when the extracts were administered to mice, serum cholesterol levels were not significantly lowered.

An eritadenine-enriched extract was easily obtained with conventional methods from shiitake mushrooms but also from other mushrooms species e.g. *Marasmius oreades*. This metabolite was partially degraded by conventional cooking technologies and scavenged by modern culinary texturizers being grilling and gelling the treatments that improved its bioaccessibility. When rats were fed the extract (21 mg/kg/day) for 35 days, eritadenine was detected in rats liver indicating that it was bioavailable and no organ damage or metabolic disorder was noticed while the atherogenic index (total cholesterol/HDL-cholesterol) was reduced.

Shiitake β -D-glucans, chitins and other water soluble polysaccharides were extracted using microwave-assisted (MAE), ultrasound-assisted (UAE) and subcritical water extractions (SWE) as well as a solid/liquid extraction unit coupled to pressure-driven crossflow filtration membranes. SFE and combinations of the indicated technologies were also tested to improve polysaccharides extractions. A response surface study indicated that the highest polysaccharide yield obtained from shiitake using MAE was 9.2%. According to fluorimetric determinations, the obtained extracts contained (1 \rightarrow 3)- β -D-glucans but they could not be properly quantified because according to NMR analysis, the extracts contained other polysaccharides that might interfere with the fluorophore used for their determination (depending on the mushroom species). UAE and SWE extractions showed similar yields (6.1 and 11.3%) and also contained (1 \rightarrow 3)- β -D-glucans and (1 \rightarrow 3),(1 \rightarrow 6)- β -D-glucans but combinations of SFE+UAE, SFE+SWE or UAE+SWE were more effective for polysaccharide extractions yielding up to 17.2% of polysaccharides. The latter extracts showed *in vitro* ability to inhibit the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), the key enzyme in the endogenous cholesterol biosynthesis. They also decreased the secretion of pro-inflammatory cytokines in LPS-activated macrophages (THP-1 cells) acting as immunomodulatory extracts.

Moreover, high amounts of a hot water soluble β -D-glucan-enriched extract were prepared using a pilot scale solid/liquid reactor and after testing microfiltration, nanofiltration and reverse osmosis. The filtration membrane showing higher filtration flux and values of bioactive compounds in the obtained extract was Nanomax50. In addition, 3 particular glucans were also isolated from the fraction remaining in the reactor because they showed HMGCR inhibitory and antioxidant capacities, as well as immunomodulatory and antitumoral properties tested in cell cultures. Therefore, both hot water and insoluble fractions were pooled together and mixed with a cold water soluble fraction (containing eritadenine and water-soluble β -D-glucans) and their hypocholesterolemic activity was tested *in vitro* and *in vivo*. The so called β -D-glucan-enriched (BGE) mixture showed the ability to displace cholesterol from dietary mixed micelles and inhibit HMGCR *in vitro* and it also lowered cholesterol levels in serum of hypercholesterolemic mice when administrated for 5 weeks. Thus, a sequential extraction procedure was designed to obtain several bioactive fractions

from the same shiitake batch. The generated fractions contained different levels of bioactive compounds such as ergosterol, eritadenine, β -D-glucans, chitins, hypotensive peptides, antioxidant phenols and anti-thrombotic lenthionine. The protocol combined 3 scalable methods using the material remaining after one extraction to generate other fractions.

Finally, a randomized, controlled, double-blind, and parallel clinical trial was carried out with 52 subjects with not treated mild hypercholesterolemia that consumed the BGE mixture or a placebo incorporated in 3 different commercial creams. After 8 weeks intervention, no significant lowering cholesterol levels nor modulation of the immune system were noticed. However, consumption of the BGE mixture was safe, volunteers achieved the dietary fiber intake recommended by authorities as cardiovascular protective diet and their colonic microbiome was differently modulated compared to placebo. Some genera positive- or negatively correlated with biomarkers related to cholesterol homeostasis. Nevertheless, the precise significance of this differential modulation could not be fully elucidated. Perhaps, longer intervention time was needed to achieve statistical significances.

Resumen

En la actualidad, el consumo de hongos comestibles es recomendado por nutricionistas y autoridades sanitarias, no solo por su valor culinario y nutricional sino también por su contenido en moléculas bioactivas que pueden resultar beneficiosas para la salud humana. La seta shiitake (*Lentinula edodes*) merece especial atención, ya que presenta una gran diversidad de actividades, tales como hipocolesterolémica, inmunomodulatoria, antioxidante, antitumoral, antihipertensiva, etc. Estas propiedades biológicas se han relacionado con compuestos específicos, por ejemplo esteroides fúngicos (ergosterol y derivados), polisacáridos (α - y β -D-glucanos, quitinas, etc.), proteínas/péptidos y metabolitos secundarios (compuestos fenólicos, eritadenina, lentinina, etc.). Por ello, el objetivo de esta tesis doctoral fue evaluar el empleo de diferentes tecnologías avanzadas de extracción que pudieran ser escalables y aplicables de forma secuencial o combinadas para obtener ingredientes funcionales a partir de setas shiitake que puedan dar lugar al diseño de nuevos alimentos funcionales.

Se ajustaron procedimientos utilizando CO₂ supercrítico (SFE) a escala piloto y gran escala para obtener extractos ricos en esteroides fúngicos. Estos extractos pudieron transformarse en fracciones ricas en ergocalciferol (vitamina D₂) sometidos a radiación UV. Los extractos supercríticos contenían hasta un 53% de ergosterol y lograron desplazar el colesterol de las micelas mixtas de la dieta utilizando un modelo de digestión *in vitro*. También fueron efectivos cuando se microemulsificaron y cuando se mezclaron con β -D-glucanos fúngicos. Sin embargo, cuando los extractos fueron administrados a ratones, no redujeron significativamente sus niveles séricos de colesterol.

Se obtuvo un extracto rico en eritadenina mediante el empleo sencillo de métodos convencionales a partir de setas shiitake pero también a partir de otras especies de setas, por ejemplo, *Marasmius oreades*. Este metabolito fue parcialmente degradado por las tecnologías de cocina convencional y secuestrado por los texturizadores utilizados en la cocina moderna, siendo el cocinado a la plancha y la gelificación los tratamientos que mejoraron su bioaccesibilidad. Cuando el extracto fue administrado a ratas (21 mg/kg/día) durante 35 días, se detectó eritadenina en el

hígado de las ratas, indicando que era biodisponible. Además, no se observaron daños en ningún órgano ni ningún desorden metabólico, mientras que se redujo el índice aterogénico (colesterol total/colesterol-HDL).

Se extrajeron β -D-glucanos, quitinas y otros polisacáridos solubles en agua utilizando extracciones asistidas por microondas (MAE), por ultrasonidos (UAE) y extracciones con agua subcrítica (SWE), así como a través de una unidad de extracción sólido/líquido acoplada a un equipo de filtración tangencial por membranas impulsado a presión. Se evaluaron las técnicas de SFE y las combinaciones de las tecnologías indicadas para mejorar la extracción de polisacáridos. Un estudio de superficie de respuesta indicó que el mayor rendimiento de polisacáridos que se obtuvo utilizando MAE fue del 9,2%. De acuerdo con las determinaciones fluorimétricas, los extractos obtenidos contenían (1 \rightarrow 3)- β -D-glucanos, aunque estos no pudieron ser cuantificados apropiadamente, ya que, según los análisis de resonancia magnética nuclear, los extractos contenían otros polisacáridos que podrían interferir con el fluoróforo utilizado para su determinación (dependiendo de la especie de seta). Las extracciones UAE y SWE mostraron rendimientos similares (6,1 y 11,3%), conteniendo también (1 \rightarrow 3)- β -D-glucanos y (1 \rightarrow 3),(1 \rightarrow 6)- β -D-glucanos, pero las combinaciones de SFE+UAE, SFE+SWE o UAE+SWE fueron más efectivas para la extracción de polisacáridos alcanzando rendimientos de hasta el 17,2%. Estos últimos extractos mostraron la habilidad de inhibir *in vitro* la 3-hidroxi-3-metilglutaril coenzima A reductasa (HMGCR), la enzima clave en la biosíntesis endógena de colesterol. También redujeron la secreción de citoquinas pro-inflamatorias por parte de macrófagos activados con LPS (células THP-1), actuando como extractos inmunomoduladores.

Además, se prepararon grandes cantidades de extractos solubles en agua caliente ricos en β -D-glucanos, a través del uso de un reactor sólido/líquido a escala piloto y después de evaluar el empleo de técnicas de microfiltración, nanofiltración y ósmosis inversa. La membrana Nanomax50 fue la que mostró un mayor flujo de filtración y los valores más altos de compuestos bioactivos en el extracto obtenido. Por otra parte, tres glucanos concretos fueron aislados de la fracción remanente en el reactor, puesto que mostraron capacidad inhibidora de la HMGCR y antioxidante, así

como propiedades inmunomodulatorias y antitumorales estudiadas en cultivos celulares. Por ello, tanto las fracciones solubles e insolubles en agua caliente fueron mezcladas junto con una fracción soluble en agua fría (que contenía eritadenina y también β -D-glucanos solubles en agua) y se evaluó la actividad hipocolesterolémica de la mezcla, tanto *in vitro* como *in vivo*. La mezcla llamada extracto rico en β -D-glucanos (BGE) mostró la capacidad para desplazar el colesterol de las micelas mixtas de la dieta y para inhibir la HMGCR *in vitro* y también redujo los niveles de colesterol sérico de ratones hipocolesterolémicos tras su administración durante 5 semanas. Entonces, un procedimiento de extracción secuencial fue diseñado para obtener varias fracciones bioactivas del mismo lote de shiitake. Las fracciones generadas contenían diferentes niveles de compuestos bioactivos como ergosterol, eritadenina, β -D-glucanos, quitinas, péptidos hipotensivos, fenoles antioxidantes, lenthionina antitrombótica. El protocolo combinó tres métodos escalables que utilizaban el material remanente después de una extracción para generar otras fracciones.

Finalmente, un ensayo clínico aleatorizado, controlado, doble ciego y paralelo fue llevado a cabo en 52 sujetos con hipercolesterolemia moderada y no tratada que consumieron la mezcla BGE o un placebo incorporados en tres cremas comerciales diferentes. Después de 8 semanas de intervención, no se detectó reducción en los niveles de colesterol ni modulación del sistema inmune de los pacientes. No obstante, el consumo de la mezcla BGE fue seguro, los voluntarios alcanzaron la ingesta de fibra dietética recomendada por las autoridades para una dieta cardiosaludable y su microbioma colónico fue modificado de forma diferente en comparación con el placebo. Se pudieron establecer diversas correlaciones positivas y negativas entre los distintos géneros y los marcadores biológicos relacionados con la homeostasis de colesterol. Sin embargo, el significado preciso de esta modulación diferencial no pudo ser completamente dilucidado. Es posible que se requiera un mayor tiempo de intervención para lograr diferencias estadísticamente significativas.

General Introduction



General introduction

Oriental cultures have consumed for centuries specific plants and mushrooms as therapeutic remedies against certain diseases. Several mushroom varieties are nowadays being scientifically tested and many of them confirmed indeed the presence of bioactive compounds with different functionalities. Therefore, at the present, novel extraction or isolation procedures are being investigated to obtain enriched bioactive fractions to design effective functional foods [1]. Although the white button mushroom (*Agaricus bisporus*) leads the Western mushroom market, other species are attracting consumers attention. Among these species, shiitake mushrooms (*Lentinula edodes*) should be highlighted due to their growing importance as source of nutrients and bioactive molecules, as well as delicatessen foodstuff for numerous culinary preparations. It has been cultivated in Asia for over a century, but its expansion in Western countries is relatively recent. Nowadays, it is the leading cultivated mushroom in the world [2].

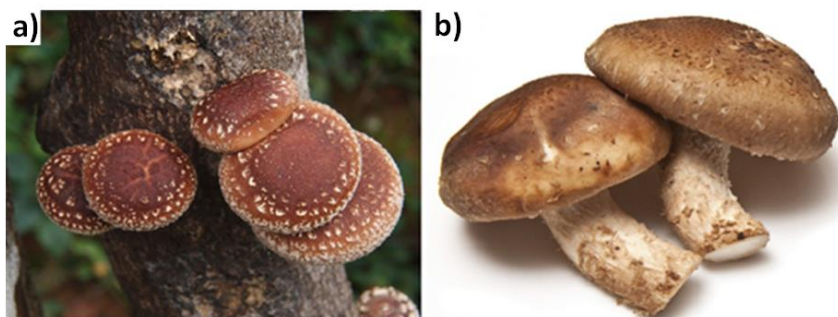


Figure 1. Shiitake mushrooms growing naturally in a shii tree (a) and after harvesting (b).

L. edodes is an originary Japanese mushroom that was named *shiitake* because it grew on natural logs of the shii trees (*Castanopsis cuspidate*) [3] (Figure 1a). Shiitake fruiting bodies are characterized by caps averaging 10-20 cm diameter and thin but hard stipes. The caps are umbrella-shaped, light to dark brown-colored

and with cream-colored flesh in the inside, besides the white gills that are placed in the inferior side. The fibrous stipes are greyish to light brown-colored (Figure 1b).

It is also known as ‘black forest mushroom’ and it is the most popular fungus cultivated not only in Japan but also in China and other Asian countries. Two main reasons are crucial to explain its popularity: on the one hand, its unique flavor and taste provided by some characteristic molecules; on the other, the medicinal features of this species that were already reported centuries ago by doctors during the Ming Dynasty (1368-1644) in China [4]. They are commonly utilized in many gastronomy preparations and usually served steamed, sautéed, fried, boiled and being part of typical soups and Asian dishes (*miso*, *dashi*, etc.) (Figure 2). Since shiitake stipes are too hard and non-palatable, only the caps are used in these meals.



Figure 2. *Miso* soup with shiitake mushrooms.

Shiitake mushrooms were firstly harvested from woods since they grew in the tree trunks and between 17th and 18th centuries, Japanese and Chinese farmers started to cultivate them. First, they collected wood logs and controlled environmental conditions for mycelial growth and fruiting events and later, in the 20th century, they started to place the substrate in plastic bags shortening the time of cultivation and increasing production yields. There are many methods that are being utilized, such as those using bottles, bricks, artificial logs, sawdust and cereal straws, coffee wastes and most of them are reproducing the natural conditions of shiitake growing in the forest [4].

1. Edible mushrooms and *Lentinula edodes* production in the world

Edible mushrooms consumption has significantly increased in the last decades. Since the late 1970s, it has been more than 30-folded. This rising trend is not only due to population growth, and this fact is evidenced with data of mushrooms consumption *per capita*, that were about 1 kg/person/year in 1997 and nowadays are approximately 5 kg/person/year [2]. China is the main producer of edible cultivated mushrooms, reaching 769 thousand tons in 2017 according to FAOSTAT estimations [5], followed by USA with 421 thousand tons. The other top countries are European: Poland, Netherlands and Spain, with respectively 303, 300 and 159 thousand tons. In Spain, the exponential growth of mushroom production is also obvious, although, except for 2015 values, there are no significant differences in recent years (Figure 3).

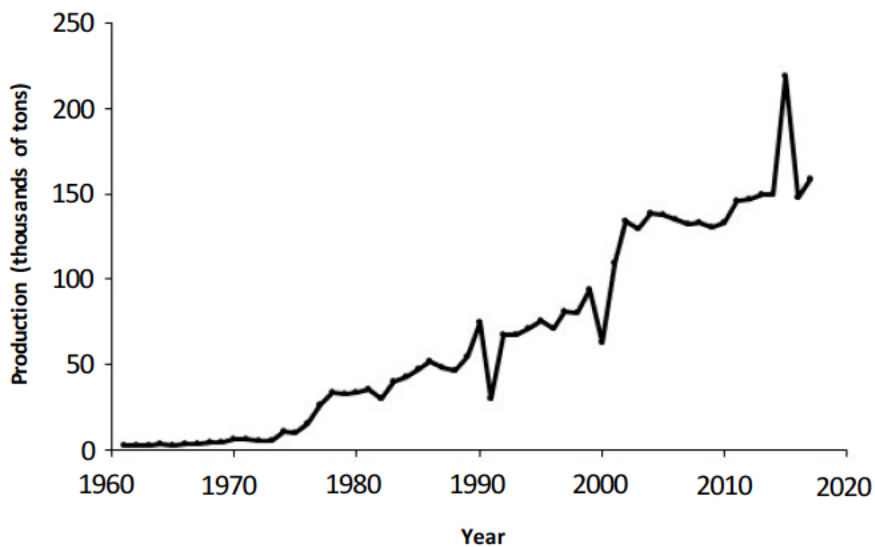


Figure 3. Cultivated mushrooms production in Spain in the last five decades. Data were obtained from FAOSTAT (2018) [5].

Spanish crops, as well as other Western countries such as the USA, are mainly focused on white button mushroom (*Agaricus bisporus*) cultivation. In fact, *Agaricus bisporus* was reported as the world's leading species in 2010 and the genus *Agaricus* was also on the top, resulting in the 30% of the world's cultivated

mushrooms [6]. However, a few years later, the same authors observed a large increase in *L. edodes* consumption placing this species as the world most cultivated mushroom nowadays [2]. Therefore, *Lentinula* is the major genus, implying 22% of cultivated mushrooms and *Agaricus* genus reduced its percentage to 15%. Surprisingly, this genus ranks fourth after not only *Lentinula*, but also *Pleurotus* (19%) and *Auricularia* (18%). The *top 5* includes *Flammulina* (11%).

Shiitake was mainly consumed in Asia in the past century but it has been introduced also in the Western market and more and more people demand these mushrooms in the USA and Europe, being also cultivated in several regions of Spain where La Rioja and La Manchuela (Cuenca and Albacete) are the major producers, although several companies are also located in different areas such as Asturias, Basque Country, etc.

2. Mushrooms as functional foods

The European Food Safety Authority (EFSA) defines the term ‘functional food’ as a ‘food that beneficially influences one or more target functions in the body beyond adequate nutritional effects; its effect either improves the health state and wellbeing and/or reduce the risk of disease’. Similar definitions were previously drawn by equivalent authorities in other countries such as the Japanese government by their FOSHU implementation and by the Food and Drug Administration (FDA) in the USA. Therefore, conventional foods with high quantities of bioactive compounds can be considered as functional foods, as well as food enriched or fortified with bioactive molecules and/or subjected to removal of undesirable compounds [7]. The term ‘functional food’ is usually misused and confused with other concepts such as ‘nutraceuticals’ or ‘food supplements’ whose forms are pills, tablets, syrups, capsules instead of conventional foods that can be daily consumed [8].

A large diversity of functional foods can be found in European market, e.g. products enriched or fortified with dietary fiber (cereals, biscuits, bread, etc.), omega-3 and omega-6 fatty acids (butter, margarine, eggs, etc.), plant sterols (butter, yogurts, drinks), probiotics (yogurts, juices, drinks), etc. (Figure 4). The inclusion of a health claim approved by the EFSA is crucial for the development of these products and

their potential commercialization [9]. Some examples of those approved claims are: barley β -D-glucans can lower blood cholesterol [10]; plant sterols can lower blood LDL-cholesterol [11]; vitamin A, B₆, B₁₂, C might contribute to the normal function of immune system [12-15]; pectins consumption with a meal might contribute to the reduction of postprandial glycemic response [16], etc.



Figure 4. Examples of functional foods commercialized in Spain.

In Europe, although some nutraceuticals and food supplements included mushrooms in their beneficial formulations, no functional foods containing mushrooms or mushroom ingredients can be found in the European market. Medicinal mushroom extracts such as *Agarikon.1*, obtained from *A. blazei*, *L. edodes* and *Ganoderma lucidum*, are sold in Europe. Other nutraceuticals such as *Grifola frondosa*, *Agaricus blazei* and *Pleurotus ostreatus* extracts are also in the market claiming immune system enhancing properties and beneficial effects against breast cancer cell. Besides, a preparation called *Mykoprotect.1* is a mixture of *L. edodes* and *G. lucidum* extracts apparently with antiviral properties [17]. However, none of these formulas bear an official EFSA approved health claim.

In contrast to Europe, the use of mushrooms in novel functional food designs is popular in Asia. Proof of this are the numerous patents that were published by Asian authors in recent years (Table 1). The patented foods ranged from the use of mushroom as flavor enhancers (e.g. meat products with edible mushrooms flavor) to their use as functional ingredient (e.g. functional salts using shiitake mushrooms, rice mixed with edible mushroom powder, etc.).

Table 1. Recent patent publications describing novel functional foods based on mushrooms ingredients (from World International Property Organization (WIPO) and Espacenet databases).

International number	Publication date	Country	Description	Ingredients
CN106490490A	2017	China	Meat product (sausage) with edible mushrooms flavor	<i>G. lucidum</i> , <i>Hericium erinaceus</i> , <i>A. blazei</i> and other edible mushrooms
CN104082819B	2016	China	Functional solid beverage based on mushrooms	<i>Coprinus</i> spp., <i>Agaricus</i> spp., <i>Ganoderma</i> spp., soybean, corn powder
KR101243168B1	2013	South Korea	Functional salt using <i>L. edodes</i> mycelium	<i>L. edodes</i>
KR20110058041A	2012	South Korea	Functional food based on mushroom and ginseng extracts	<i>Phellinus linteus</i> and ginseng
KR101027964B1	2011	South Korea	Functional food based on brown rice with medicinal mushrooms	<i>Phellinus</i> spp., <i>Inonotus obliquus</i> , <i>Ganoderma</i> spp., rice
KR101012405B1	2011	South Korea	Functional food based on mushroom mycelium fermentation	<i>G. lucidum</i> , <i>Agaricus</i> spp. and other mushrooms, rice flour, algae extract, fish extract
KR1020080100466A	2010	South Korea	Functional tea or pasta based on a mixture of <i>Flammulina velutipes</i> and blue crab	<i>F. velutipes</i> , blue crab

The USA is following the trend of Asian countries and some products are recently commercialized bearing health claims because of the mushrooms included as bioactive ingredients in their formulation. For instance, edible mushrooms were used to prepare a *mushroom coffee* that combines coffee grains with some mushroom species such as *I. obliquus* or *H. erinaceus* (Figure 5).

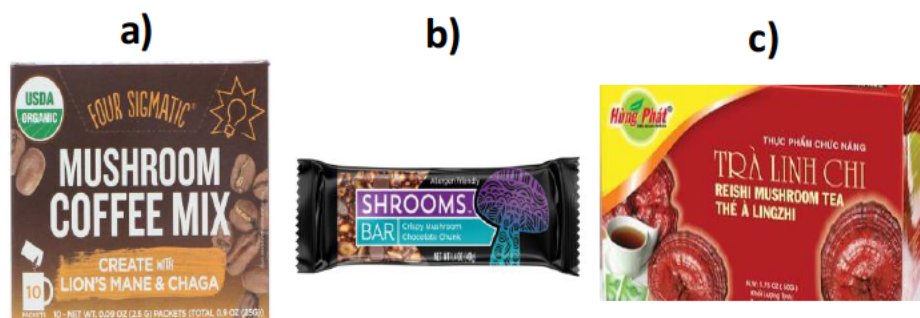


Figure 5. Commercialized foods with mushroom ingredients: a “mushroom coffee” combining coffee grains with *H. erinaceus* and *I. obliquus* (a); an *A. bisporus* snackbar (b); and a *G. lucidum* tea box (c).

Other novel popular mushroom-based food are snack bars, teas, cheeses, breads (mixing cereal flour with mushroom powder) and recently cereals and flakes were designed including mushroom powder in their composition [18].

3. Bioactive compounds from *Lentinula edodes*

In general, edible mushrooms provide a high nutritional value since they contain a high moisture percentage, significant levels of carbohydrates and proteins and low fat amounts. Particularly *L. edodes* showed a moisture content of approx. 90% fresh weight of the fruiting body and the reported values for its main constituents were: 68-84% total carbohydrates (dry weight), 10-20% total proteins, 1-8% fat and 3-5% ash [19-22]. The indicated differences usually depend on the analyzed strain, location, cultivation and harvesting conditions, storage, etc.

When compared to the common white bottom mushroom (*A. bisporus*), shiitake fruiting bodies usually contained higher percentages of carbohydrates and lower of proteins (Table 2). However, the range of these values within a single strain can be so wide that it is difficult to point the one with higher concentrations of a nutrient compared to the rest of species. Nevertheless, they all contained mainly carbohydrates and large concentrations of proteins.

Table 2. Proximate composition of commonly consumed edible mushrooms (% dry weight).

Species	Carbohydrate (%)	Protein (%)	Fat (%)	Ash (%)	References
<i>Lentinula edodes</i>	68-84	10-20	1-8	3-5	[19-22]
<i>Agaricus bisporus</i>	49-62	21-27	2-4	9-11	[22-24]
<i>Pleurotus ostreatus</i>	61-74	16-23	1-5	6-10	[22, 25-27]
<i>Boletus edulis</i>	31-71	18-29	4-6	5-9	[22, 28-30]
<i>Flammulina velutipes</i>	56-86	19-27	3-9	6-9	[22, 24, 26, 31]
<i>Agrocybe cylindracea</i>	66-73	16-22	3-4	7-8	[22, 32]
<i>Lactarius deliciosus</i>	25-67	20-30	2-8	5-11	[27, 32, 33]

Moreover, shiitake mushrooms also contain other nutritionally important compounds such as vitamins from B complex and vitamin C (approx. 0.2 mg/g) [19, 34]. Vitamin D₂ content is usually low since shiitake fruiting bodies are cultivated in dark but it might be higher in mushrooms from the woods that are exposed to sun light [35]. Shiitake also contains trace elements and minerals being potassium the most abundant but also significant amounts of magnesium, sodium, calcium, iron, manganese, zinc, phosphorus and cadmium were detected [19, 21, 36]. However, shiitake and other edible mushrooms are nowadays under investigation because besides these compounds, they contain others that might act as functional ingredients since they show interesting biological activities beneficial for human health.

3.1. Fungal sterols and ergocalciferol

The major sterol that can be found in mushrooms is ergosterol (ergosta-5,7,22-trien-3 β -ol) (Figure 6) that, together with ergosterol peroxide (5 α ,8 α -epidioxi-22 E -ergosta-6,22-dien-3 β -ol) and other structurally related derivatives, demonstrated *in vitro* relevant biological properties such as hypocholesterolemic, antitumoral, antioxidant and anti-inflammatory [37, 38].

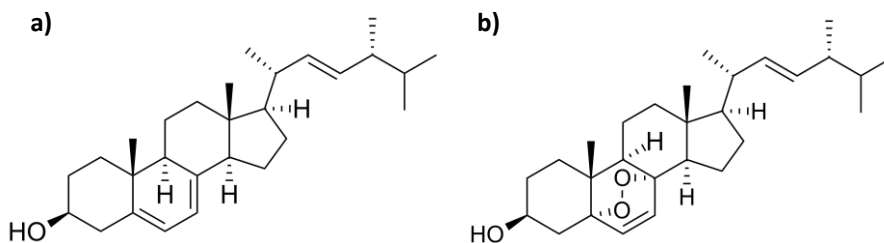


Figure 6. Molecular structures of ergosterol (a) and ergosterol peroxide (b).

Ergosterol is the main sterol constituent of fungal membranes and therefore it is present in all mushroom species including shiitake. This compound was found in concentrations ranging from 0.2 to 12.1 mg/g, however, the specific amount mainly depends on strain and developmental state and, therefore, it is used as biomarker for proper mycelial growth [39]. Ergosterol is synthesized when mycelium is growing and fruiting bodies are increasing their size and its synthesis is stopped during senescence or stressing conditions. Thus, ergosterol levels in *L. edodes* might range from approx. 3.5 to 6.8 mg/g (dry matter) [40-42]. These levels were slightly higher than those reported for *B. edulis* (4.0-4.9 mg/g) or *F. velutipes* (0.7-4.5 mg/g) and similar to other species such as *A. bisporus* (5.0-8.9 mg/g) and *P. ostreatus* (6.8-12.1 mg/g) [40, 41, 43].

Mushrooms contain other sterol derivatives such as ergosta-7,22-dienol, ergosta-5,7-dienol, ergosta-7-enol (fungisterol), ergosta-5,8,22-trien- β 3ol, lanosterol, etc., differing in concentrations depending on the species considered. In closely related organism such as truffles, brassicasterol was also present in concentrations close to ergosterol [44]. In *L. edodes*, lower fungisterol concentrations (0.6-1.8 mg/g) than ergosterol were reported, but similar to other derivatives such as ergosta-7,22-dienol (0.2-1.7 mg/g) and ergosta-5,7-dienol (0.3-1.4 mg/g). These sterols were also noticed in species such as *P. ostreatus* (0.2-1.3 mg/g) and *A. bisporus* (0.2-0.9 mg/g) and they were found in higher amounts in others such as *B. edulis* and *F. velutipes* (2.2-3.2 and 1.1-3.0, respectively) [41, 43].

Ergosterol is also the vitamin D₂ (ergocalciferol) precursor and it can be transformed into the vitamin by UV-irradiation methods [35]. Irradiated ergosterol changed first into previtamin D₂ and then it is rearranged to vitamin D₂ [45] although

side reactions might take place generating other isomers such as lumisterol₂ and tachysterol₂ (Figure 7). Vitamin D₂ is not only involved in osseous system maintenance and calcium metabolism, but also in many crucial health features, *e.g.* immune system function or cardiovascular health [46].

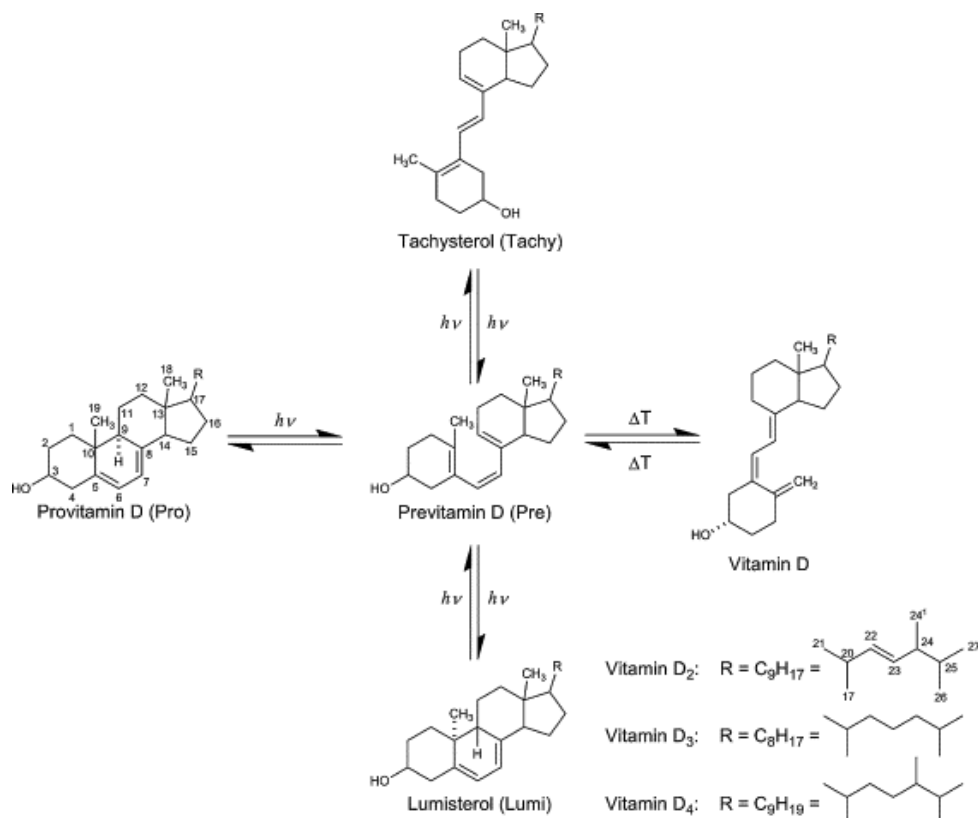


Figure 7. Provitamin D isomerisation after UV exposure (from Wittig et al. (2013) [45]).

On the other hand, ergosterol structure is so similar to cholesterol that it could compete with the animal sterol for its incorporation in dietary mixed micelles, reducing cholesterol absorption by a similar mechanism to the one proposed for phytosterols. He et al. (2019) [47] observed that commercial ergosterol was able to impair cholesterol inclusion to micelles *in vitro* and noticed a decrease in cholesterol serum levels in rats, as well as an increase in fecal cholesterol. Gil-Ramirez et al. (2014) [48] used an *in vitro* digestion model and also found a reduction in cholesterol content inside the micelles after ergosterol addition. Later on, Gil-Ramirez et al.

(2016) [49] evaluated this compound in a mice model leading to a decrease in the atherogenic index (total cholesterol/HDL-cholesterol). Moreover, ergosterol modulated the expression of genes related to the cholesterol homeostasis as hypocholesterolemic drugs such as simvastatin and ezetimibe in a mice model. Similarly, when applied to HepG2 cells, ergosterol containing samples up-regulated the LDL-receptor and modulated other genes related to lipid metabolism. In addition, ergosterol showed ability to inhibit DHCR24 (delta24-sterol reductase), the enzyme that catalyzes the reduction of the double bond at C-24 in the cholesterol biosynthesis pathway [50].

Anti-diabetic properties were also attributed to ergosterol isolated from *P. ostreatus* when administered to mice with spontaneous type-2 diabetes mellitus. The hypoglycemic proposed mechanism involved the stimulation of GLUT4 expression and translocation, a protein that plays a key role in glucose homeostasis [51]. Antioxidant capacity was also reported not only for ergosterol, that was able to inhibit lipid peroxidation [52] but also for other fungal sterols including a novel identified sterol derivative (cerevisterol 6-cinnamate) isolated from *H. erinaceus* [53].

Other derivatives such as ergosterol peroxide showed interesting biological properties. Kobori et al. (2007) [54] noticed anti-inflammatory activities when isolated it from *Sarcodon asparatus* and this compound also exerted antitumor effects on ovarian, hepatocellular carcinoma and colon cancer cells [54-56]. Moreover, two ergostane-type sterols isolated from *Pleurotus eryngii* were also pointed as candidates against breast cancer growth [57].

3.2. Eritadenine

Eritadenine (2(*R*),3(*R*)-dihydroxy-4-(9-adenyl)butanoic acid) (Figure 8), also named lentysine or lentinacin, is an adenosine analog derived from secondary metabolism. It was firstly isolated from *L. edodes* [58] and it was not studied in other mushrooms for many years. However, later on, it was detected in a few other mushrooms, although eritadenine levels were always higher in *L. edodes* (6.2-6.4 mg/g). Other species such as *F. velutipes* or *A. bisporus* presented significant amounts

(4.3 and 3.8 mg/g, respectively) while *P. ostreatus* or *G. lucidum* showed very low eritadenine concentrations (2.1 and 0.2, respectively) [59, 60].

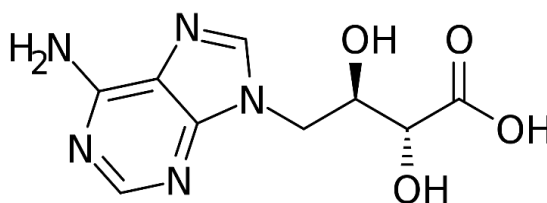


Figure 8. Molecular structure of eritadenine.

Eritadenine showed hypocholesterolemic effects although its mechanism of action remains unclear. This metabolite was able to inhibit the enzyme S-adenosyl-L-homocysteine hydrolase, modifying the hepatic metabolism of phospholipids. The increase of S-adenosyl-L-homocysteine inhibited phosphatidylethanolamine N-methylation, decreasing the ratio phosphatidylcholine/ phosphatidylethanolamine. Moreover, the suppression of S-adenosyl-L-homocysteine hydrolase activity correlated with lower plasma homocysteine concentrations. Fukada et al. (2006) [61] observed that eritadenine supplementation could revert the hyperhomocysteinemia induced by guanidinoacetic acid in rats, decreasing cardiovascular risk. However, the exact implication of eritadenine in lowering plasma cholesterol levels was not fully elucidated. Takashima et al. (1973) [62] suggested that eritadenine might develop its hypocholesterolemic action by decreasing lipoprotein-cholesterol secretion from the liver into the blood and/or increasing cholesterol uptake by tissues from plasma, being related with changes in membrane phospholipids composition. Nevertheless, later on, Sugiyama & Yamakawa (1996) [63] reported that the hypocholesterolemic effects could be related with alterations of plasma phosphatidylcholine species composition. Shimada et al. (2003) [64] also observed that eritadenine suppressed $\Delta 6$ -desaturase activity and inhibit linoleic acid metabolism, although its correlation with lowering plasma cholesterol levels remains still unknown.

Gil-Ramirez et al. (2016) [65] noticed that an eritadenine-rich extract from *L. edodes* was able to modulate expression of genes involved in the cholesterol metabolism. However, when pure eritadenine standard was tested *in vitro*, no inhibition of HMGCR activity was observed. Moreover, Yang et al. (2013) [66]

administered eritadenine and *L. edodes* to hypercholesterolemic mice and decreases in serum lipid levels, hepatic fat accumulation and aortic atherosclerotic plaque formation were recorded.

In addition, Afrin et al. (2016) [60] reported *in vitro* antihypertensive activity for eritadenine purified from *L. edodes*, since it was able to inhibit angiotensin-converting enzyme (ACE).

3.3. D-Glucans and chitins

Among mushroom carbohydrates, glucans must be highlighted because of their beneficial properties. According to their anomericity, these homopolysaccharides could be classified as α -D-glucans and β -D-glucans, and also mixed α/β -D-glucans [67].

Table 3. β -, α -D-glucans and chitins content in commonly consumed edible mushrooms (% w/w dry weight).

Mushroom species	β -D-glucans (% w/w)	α -D-glucans (% w/w)	Chitins (% w/w)	References
<i>Lentinula edodes</i>	20-33	1-2	3-5	[68-71]
<i>Agaricus bisporus</i>	9-13	2-5	8-10	[68-71]
<i>Pleurotus ostreatus</i>	24-40	1-8	2-5	[68-71]
<i>Pleurotus eryngii</i>	15-44	4-10	3-4	[68, 70]
<i>Boletus edulis</i>	17-58	3-5	-	[69]
<i>Flammulina velutipes</i>	20-21	-	9-10	[70, 72]
<i>Craterellus cibarius</i>	13-27	1-2	-	[68, 73]
<i>Grifola frondosa</i>	33-34	-	1-2	[70, 74]

β -D-glucans are normally present in edible mushrooms in higher amounts than α -D-glucans, ranging between 9-58% w/w (dry weight) (Table 3). *L. edodes* usually contained higher levels (20-33%) than other species such as *A. bisporus* and *F. velutipes* (9-13 and 20-21 %, respectively), but lower than others such as *B. edulis* (up to 58 %) [68, 69].

Most of the works were carried out to study β -D-glucans structures and functions. They showed different structures (Figure 9) when compared to the well-known cereal β -D-glucans, since fungal β -D-glucans are constituted by a main linear glucose chain with (1 \rightarrow 3)- β -linkages and (1 \rightarrow 6)- β -branches. These compounds showed many and varied biological activities such as hypocholesterolemic, antioxidant, antitumoral, antimicrobial, immunomodulatory, anti-inflammatory, hypoglycemic, etc. [75].

Several mechanisms of action were postulated to explain the hypocholesterolemic properties of β -D-glucans and some were related to their ability to increase intestinal viscosity during digestion leading to an increase in fat and cholesterol excretion. They might reduce lumen bile acids levels as suggested *in vitro* by Palanisamy et al., 2014 [76]. Apparently, β -D-glucan-enriched fractions from *L. edodes* and other mushrooms such as *A. bisporus* and *P. ostreatus* could scavenge bile acids into their structure. Their forced excretion would reduce their intestinal re-absorption and transport to the bloodstream inducing cholesterol transformation into bile acids to reestablish their balance reducing cholesterol levels. Other reports indicated that when β -D-glucans reach the large intestine they might be used by colonic microbiota to produce short-chain fatty acids that might be able to impair endogenous cholesterol biosynthesis [75].

Antioxidant activities were also observed for mushroom β -D-glucans, particularly for those isolated and well characterized such as schizophyllan from *Schizophyllum commune* or pleuran from *P. ostreatus* [77, 78]. The latter polysaccharide also validated its antioxidant properties *in vivo* using male Wistar rats. However, there are very limited studies describing the antioxidant activity of *L. edodes* glucans, only the work of Li et al. (2019) that recently isolated an (1 \rightarrow 6)- β -D-glucan acting as hydroxyl radical scavenger [79].

The most studied β -D-glucan from shiitake is lentinan and, although it also showed antioxidant properties, its antitumoral capacity must be highlighted. Despite the fact that most of the works claimed *in vitro* effects [80], Ng & Yap (2002) [81] reported significant regression of tumor formation in leukemia AKR mice after lentinan oral administration. However, the mechanism of action when administrated as feed is not fully elucidated since lentinan is often intravenously applied to exert the

antitumoral effect. Moreover, other reports indicated other activities such as immunomodulatory and antiviral [82, 83].

Antitumoral activities of mushroom glucans are usually explained by their effect as immune system stimulators that diminishes tumour resistance [84, 85, 86]. However, other works demonstrated that certain β -D-glucans, for instance, (1 \rightarrow 3),(1 \rightarrow 6)- β -D-glucans isolated from shiitake were able to exert a direct cytotoxic effect on tumoral cells, besides its immunomodulatory effects [86].

A significantly lower number of studies were carried out on fungal α -D-glucans bioactivities compared to β -D-glucans. A (1 \rightarrow 3)- α -D-glucan was isolated from shiitake mushrooms showing antioxidant and antitumoral properties after the glucan was carboxymethylated to increase its water solubility [87]. Moreover, other α -D-glucans from *G. frondosa* also showed antitumoral, immunomodulatory, hypoglycemic and antioxidant effects in mice experiments [84, 88, 89].

Both α - and β -D-glucans extracted from mushrooms (e.g. *P. ostreatus* and *P. eryngii*) were also studied because, as dietary fiber compounds, they exerted prebiotic activity and effects on the modulation of gut microbiome [90].

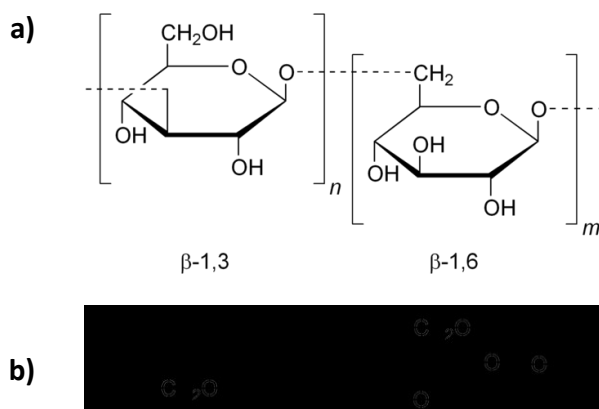


Figure 9. Typical structure of fungal β -D-glucan (a) compared to cereal β -D-glucan (b)

Chitin (Figure 10) is the second most common polymer after cellulose in nature and besides invertebrates, crustacean shells, insect cuticles and yeasts, mushrooms also contain this aminopolysaccharide [91]. Chitin is a copolymer of β -(1 \rightarrow 4)-linked D-glucosamine and N-acetyl-D-glucosamine units and when submitted to enzymatic or alkaline deacetylation, it is transformed into chitosan. In edible mushrooms, chitins levels ranged 2-10%, being higher in *A. bisporus* and *F. velutipes* (8-10 and 9-10, respectively) than in *L. edodes* and *P. ostreatus* (3-5 and 2-5 %).

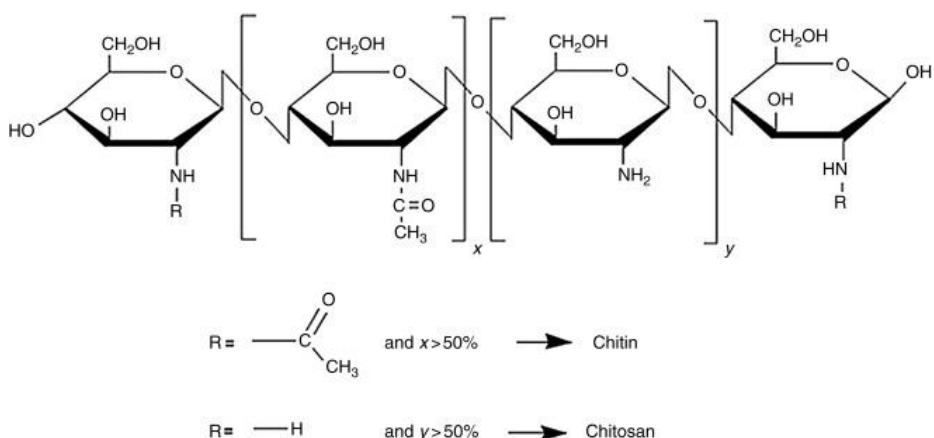


Figure 10. Chitin and chitosan structure, considering their acetylation degree. Figure from Nesic & Seslija (2017) [92].

Chitin is also considered as dietary fiber being recommended as prebiotic compound to maintain a healthy microbiome profile [90] but chitosan attracted more attention in the last decades because of its interesting physicochemical and bioactive properties. The latter compound showed hypocholesterolemic activity according to *in vitro* studies but it also demonstrated *in vivo* capacity to reduce serum cholesterol levels in animals and humans. Its mechanism of action remains still unclear although some authors suggested that its fat-binding capacity (directly related with its deacetylation degree) and its ability to increase bile-acid excretion might be involved impairing cholesterol absorption [93]. Moreover, chitosan obtained by transformation of *A. bisporus* chitins showed antimicrobial activity against *Saccharomyces cerevisiae* and *Escherichia coli* *in vitro* and when applied as coating to fresh-cut melons [94]. Chitosan from *Boletus bovinus* and *Laccaria laccata* showed *in vitro* antitumoral

activities and other chitosan derivatives obtained from different sources showed immunostimulatory and antitumor effects observed *in vitro* and *in vivo* in tests [93, 95].

3.4. Phenolic compounds

Mushrooms beneficial properties for human health are deeply influenced by their compounds from the secondary metabolism and one of the most important groups are phenolic compounds. Besides others, they were mainly studied because of their *in vitro* and *in vivo* antioxidant properties carried out by different mechanisms of action, as well as antimicrobial activity.

The precise phenolic composition of mushrooms is still questionable since there are contradictory opinions. Some authors described as major bioactive phenols compounds frequently found in plants such as gallic, protocatechuic, *p*-hydroxybenzoic, *p*-coumaric, cinnamic and caffeic acids (Figure 11) [96].

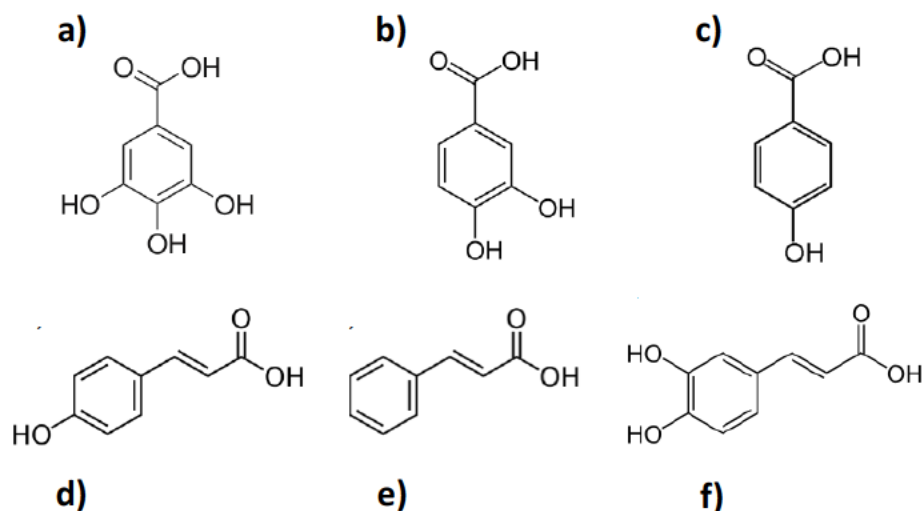


Figure 11. Phenolic compounds described in mushrooms: gallic (a), protocatechuic (b), *p*-hydroxybenzoic (c), *p*-coumaric (d), cinnamic (e) and caffeic acids (f).

However, other authors detected different phenolic compounds such as γ -glutaminy-4-hydroxybenzene (GHB), γ -glutaminy-3,4-dihydroxybenzene (GDHB) and precursors such as tyrosine that together with other derivatives (agaritin, L-DOPA, etc.) were involved in the fungal melanogenesis as substrates, inhibitors or

products of oxidative enzymes such as polyphenol oxidases or peroxidases [97]. Similarly, many publications also indicated the presence of polyphenols such as flavonoids when they are metabolites deriving from the plant secondary metabolism. But, other reports indicated that flavonoids are toxic for mushrooms and that they lack the enzymes required for their synthesis [98].

L. edodes total phenols content varied depending on the study between 9 and 13 mg/g (dry matter) [99, 100]. These values were lower than those reported for *A. bisporus* (22 mg/g) and much lower than for *B. edulis* (40 mg/g). Other mushrooms such as some belonging to the *Lactarius* or *Russula* genera showed lower concentrations than shiitake (approx. 4.5 to 3.3 mg/g) [101]. However, since most of the phenolic compounds are generated during the secondary metabolism, their levels might be highly influenced by the stress level of the mushroom being higher in fruiting bodies suffering infections or oxidations, etc. In fact, most of them are produced to act as antioxidants since they are able to donate electrons to neutralize reactive oxygen species (ROS), chelate metals such as iron or copper (that are also related with some ROS generation) and inhibit enzymes involved in oxidative and inflammatory processes (oxidases, protein kinase C, S-glutathione transferase) [102].

Water extracts obtained from *L. edodes* showed higher free radical scavenging capacity than methanol extracts suggesting that water soluble compounds were more effective antioxidants than less polar molecules. Nevertheless, in both water and organic fractions, the noticed activity correlated with their total phenols content [99]. Since many of those phenols were substrates of peroxidases and polyphenol oxidases, the antioxidant properties of the water extracts were highly influenced by the activity of these enzymes.

Similar positive correlations between total phenol contents and antioxidant activities were noticed when other edible mushrooms were studied. Fractions obtained from e.g. *A. bisporus*, *F. velutipes*, *A. blazei* [103], *Ganoderma adspersum*, *Inonotus hispidus*, *Russula chloroides*, *Sarcodon imbricatus* [104], *Craterellus cornucopioides* [105], *Gloeophyllum* spp. [106], *Tuber melanosporum* [107], *Pleurotus tuber-regium* [108], etc. showed interesting antioxidant properties depending on the method utilized to generate phenol-enriched fractions. One of these

fractions, obtained from *A. bisporus*, was tested as bioactive ingredient to supplement pasta [109]. Results indicated that the antioxidant capacity of the pasta was enhanced with the mushroom extract and it correlated with the extra amounts of phenolic compounds incorporated.

However, when Reis et al. (2012) [100] compared the antioxidant activities and total phenols content, it was found out that they were only correlating in the fruiting bodies but not in the mycelium. The lack of correlation could be due to the lower antioxidant capacity noticed for the phenolic compounds present in the mycelium compared with the high activity noticed in the sporophores. Other reports indicated that the antioxidant activity also depends on the ergothioneine (2-mercaptohistidine trimethylbethaine) concentrations, a derivative of histidine containing a sulfur atom on the imidazole ring synthesized not only in fungi but also in some bacteria [110-112]. There are works indicating that the radical scavenging activity of specific extracts from *P. ostreatus* correlated with the ergothioneine content [113] and others that found no correlation between antioxidant capacities and ergothioneine content in *Pleurotus* spp. [114].

Moreover, another role of phenols deriving from the secondary metabolism is the fungal protection against pathogens and that is the main reason for the antimicrobial activity noticed in many of the mushroom extracts including high levels of phenolic compounds. Those obtained from *L. edodes* showed interesting *in vitro* antimicrobial activities even against dangerous food contaminants such as *Bacillus cereus* and *Staphylococcus aureus* [103]. The growth of these toxic bacteria was also inhibited by phenols from other mushrooms e.g. *A. bisporus*, *F. velutipes* and *A. blazei*. The phenolic fractions obtained from *C. cornucopioides* also showed antibacterial properties not only against *B. cereus* but also against *Bacillus subtilis* [105]. The last fraction also showed antimutagenic and antitumoral properties *in vitro* indicating that fungal phenols might show many interesting biological activities. Moreover, *Gloeophyllum* spp. extracts were able to act respectively as moderate and potent acetylcholinesterase and butylcholinesterase inhibitors, postulating them as interesting candidates for further studies related with neurodegenerative diseases such

as Alzheimer's [106]. *P. tuber-regium* phenolic-enriched extracts also exerted anti-angiogenic activity *in vitro* according to Lin et al. (2015) [108].

3.5. Lenthionine

The principal aromatic substance in *L. edodes* is an organosulfur compound named lenthionine (1,2,3,5,6-pentathiepane) (Figure 12). Besides its importance in shiitake flavor, it also showed interesting bioactive properties, although not many studies were carried out.

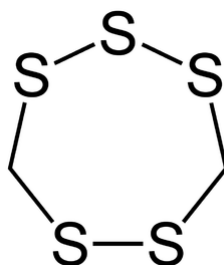


Figure 12. Molecular structure of lenthionine.

Shimada et al. (2004) [115] observed that this compound could inhibit platelet aggregation *in vitro* when it is induced by arachidonic acid ($IC_{50} = 161 \mu\text{g/mL}$) or a thromboxane A2 analog ($IC_{50} = 183 \mu\text{g/mL}$). Recently, Kupcova et al. (2018) [116] tested its *in vitro* antioxidant, antitumoral, antimicrobial and anti-inflammatory activities. Apparently, it exerted inhibitory activities against yeasts and other fungi and it reduced $\text{TNF-}\alpha$ secretion suggesting anti-inflammatory potential. However, the observed antioxidant and antitumoral effects were not validated by pure lenthionine but by using shiitake extracts and therefore its mechanisms of action are not fully determined and more studies are required to elucidate its biological properties.

3.6. Specific proteins and peptides

Mushrooms also produce bioactive proteins such as lectins, fungal immunomodulatory proteins (FIP) and other proteins with enzymatic functions such as ribosome inactivating proteins (RIP), etc. [117].

Lectins are proteins that bind specifically to cell surface carbohydrates and present the ability to agglutinate cells. They were avoided through decades because of their antinutritive and toxic character. However, recent studies describing their antitumoral activity placed them as one of the most extensively studied mushroom proteins. Specific lectins were successfully isolated and purified from *L. edodes* but their bioactivities were not deeply studied, only its hemagglutinating activities [119]. However, antitumoral properties were observed *in vitro* for lectins isolated from *Clitocybe nebularis* against human leukemic T cells [120] and those obtained from *H. erinaceus* [121], *Pholiota adiposa* [122], *Russula lepida* [123] and *Russula delica* [124]. These lectins, except those from *C. nebularis* and *R. lepida* also showed antiviral activities, particularly HIV-1 retrotranscriptase inhibitory activities. Moreover, *H. erinaceus* lectin showed mitogenic effects on mouse splenocytes. Other lectin isolated from *Sprassis latifolia* exerted *in vitro* antimicrobial activity against some bacteria (*E. coli*, *S. aureus*, *P. aeruginosa*) and fungi (*Candida* spp., *Fusarium* spp.) [125].

FIPs are a novel family of proteins with biological activities isolated from edible and medicinal mushrooms that exerted their effects on immune cells. Moreover, they showed antitumoral activities with potential for immunotherapies. Although the exact antitumoral mechanism is not fully elucidated, studies suggested that it might be related to their activation of an immune response or direct cytotoxicity against tumor cells [126]. For instance, Chang et al. (2010) [127] observed that oral administration of a well-characterized FIP from *F. velutipes* to a murine hepatoma model inhibited tumor growth and angiogenesis via IFN- γ -mediated immune responses and increased life span. Furthermore, Lin et al. (2010) [128] reported that a FIP from *Ganoderma microsporum* showed anti-metastasis effects by inhibiting migration of lung cancer cells mediated by the epidermal growth factor. Nowadays, FIPs from *L. edodes* were not investigated, although Jeurink et al. (2008) [129] observed that proteins extracted from *L. edodes* were able to downregulate pro-inflammatory cytokines secretion in human peripheral blood mononuclear cells.

In addition, marmorin, an isolated protein from *Hypsizigus marmoreus*, showed a large interest as antitumoral and antiviral compound by acting as RIP.

These proteins were specifically studied because they were able to inactivate ribosomes by deleting one or more adenosine residues from mRNA acting therefore as antitumoral proteins [130].

Peptides released after protein hydrolysis might show interesting biological activities and those derived from cereals have gained much of attention. However, fungal hydrolysates seemed to include an interesting potential too despite the short number of studies carried out until now. Farzaneh et al. (2018) [131] utilized gastrointestinal proteases (pepsin, trypsin, α -chemotrypsin) to obtain *A. bisporus* and *Terfezia claveryi* hydrolysates that exerted antioxidant and antimicrobial activities. *A. bisporus* proteins were also submitted to alkaline hydrolysis and treatment with proteases under ultra-high pressures by Zhao et al. (2017) [132] and the obtained peptides were able to activate alcohol dehydrogenase and aldehyde dehydrogenase, suggesting that these hydrolysates could be potentially used to treat alcohol intoxication. Moreover, antioxidant activities were also noticed in fungal hydrolysates according to Kimatu et al. (2017) [133].

4. Effect of processing and digestion on the fungal bioactive ingredients

The integration of a functional ingredient in a food matrix to be commercialized requests the use of machinery such as mixers, grinders and particularly heaters that might compromise the stability of the bioactive compound. Afterwards, the functional food is stored in a supermarket for a while until the client purchases it and this period might also influence the final content of the bioactive metabolite. Moreover, depending on the food matrix where the compound was integrated, it might require another heat treatment or domestic processing just before consumption. These culinary treatments might modify the molecule concentration as well.

On the other hand, the type of food matrix where the bioactive compound is incorporated might enhance or impair the assimilation of the compound during digestion facilitating or hindering its bioavailability. Particular attention should be given when the functional ingredient is presented as an innovative gastronomic

delicatessen mixed with certain additives characteristic of the so called ‘molecular gastronomy’ where modern chefs use food-grade chemical compounds to design very good-looking dishes. Furthermore, those metabolites that finally are not transported into the enterocytes and absorbed in the small intestine will reach the colonic microbiota inducing an effect that might be positive or negative. Therefore, the inclusion of a bioactive ingredient into a food product to develop a novel functional food requires many studies from the complete production chain to consumption before its introduction in the market.

4.1. Effect of processing and culinary treatments on mushroom bioactive ingredients

Heating is the process utilized in the food industry that induces higher variations not only on the nutritional value of processed foods but also on the concentrations of bioactive compounds in the final product. However, most of the works studied its effects on the main constituents or on vitamins [134-136] but not many reports were focused on the effect of the industrial treatments on the bioactive molecules from edible mushrooms and only a few of them recorded the effect of culinary heating during domestic cooking.

For instance, no studies were published describing thermal stability of ergosterol or fungal sterols during industrial or domestic processes. However, they seemed to be rather stable since the conventional analytical procedures used for their quantification imply hot alkaline extraction (80-85 °C) [41, 137], although some reports noticed ergosterol degradation e.g. in a tomato pasta serum (ergosterol indicates fungal presence) at temperatures of 70-95 °C [138]. Only a few reports can be found describing the effect of culinary treatments on vitamin D levels: Mattila et al. (1999) [136] noticed losses lower than 10% when *Cantharellus* spp. were grilled and Loznjak & Jakobsen (2018) [139] obtained similar results, since they observed losses of 12-19% when *A. bisporus* slices were grilled, that indicated lower destruction when compared to other culinary treatments such as baking or boiling, where 33 and 38% (respectively) of vitamin D was degraded.

Studies concerning eritadenine are also scarce and contradictory. On the one hand, Zhu et al. (2019) [140] indicated that eritadenine could be partially lost during the mushroom washing with tap water. But, Sanchez-Minutti et al. (2019) [141] reported that autoclaving (121 °C) or boiling did not reduce eritadenine content in *L. edodes* and higher temperatures such as those of frying or roasting were needed to reduce its levels.

Most of the previous studies investigated the thermal stability of fungal polysaccharides since water at high temperatures is frequently used for their extraction and they can influence the 3D structure and bioactivities of several particularly sensitive polysaccharides [142, 143]. A detailed study carried out on a (1→3),(1→6)-β-D-glucan isolated from *Auricularia auricula-judae* indicated that the compound maintained its conformation in hot water below 155 °C. Above this temperature, the glucan structural chain changed, intra- and interhydrogen bonds suffered dissociation above 160 °C and covalent bonds were destructed over 200 °C [144]. These results were in concordance with other where depolymerization of chitins into chitooligosaccharides was recorded when pressurized hot water (200 °C) was used [76]. However, at domestic cooking temperatures, mushroom polysaccharides are rather stable. Treatments such as boiling, microwaving and grilling did not modify the carbohydrate values in *A. bisporus*, *P. ostreatus*, *P. eryngii* or *L. edodes* mushrooms except when they were submitted to deep frying inducing an important reduction. β-D-Glucans, as the major constituents of the carbohydrate fractions, were similarly influenced [68]. These authors did not notice large differences after microwaving or grilling and only a slight increase was observed after boiling, probably due to the leaching of soluble compounds to cooking water. However, the most drastic treatment was frying where large degradation was recorded particularly in mushrooms such as *Pleurotus* spp. and *L. edodes*. Nevertheless, other works did not report significant degradation on carbohydrate content on fried *L. deliciosus*, but they noticed a 34% increase because of water loss occurring during frying [145].

Yet, the polysaccharides that are easily solubilized in water seemed to be more sensitive to heat. Those isolated from *P. ostreatus* were not affected by

blanching but boiling of the fruiting bodies for 15 min led to 35% decrease in their initial amounts [134]. Moreover, water-soluble polysaccharides were dissolved in the cooking water that is usually discarded after soaking and cooking [140].

The effect of steaming and roasting was also studied on the phenolic compounds from two mushroom varieties (*Termitomyces schimperi* and *Volvariella volvacea*) and results indicated that after steaming the mushroom contained higher levels (16 mg/g) than when they were still raw (13 mg/g) [146]. Abacan et al. (2017) [147] also noticed a significant reduction in phenolic compounds levels after boiling of some mushrooms. Manzi et al. (2004) [25] pointed also reductions of total phenols content in *Boletus* spp. (14-31%) and *Agrocybe aegerita* (5%) after grilling and Roncero-Ramos et al. (2017) [68] observed decreases after culinary treatments, particularly boiling but also frying.

Other culinary processes that might alter the concentrations of bioactive compounds that are assimilated are those utilized in the molecular gastronomy. These novel culinary procedures might influence their levels since they include food additives in their formulations (hydrocolloids, thickeners, etc.) and these compounds could modify the bioactive ingredients bioaccessibility. For instance, ‘texturization’ often utilizes agar-agar as a thickener (Figure 12). This polysaccharide is extracted from some algae genera such as *Gellidium* and it is a galactose polymer (mixture of agaropectin and agarose). Other usual method that chefs use is ‘spherification’ that is carried out with alginate, an anionic polysaccharide. A more conventional method is ‘gelling’ using gelatin, a protein that acts as a colloidal gel too.

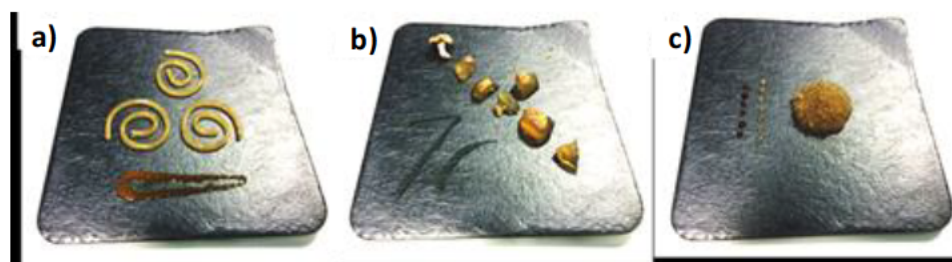


Figure 12. Shiitake powder appearances after a) thickening with agar-agar, b) gelling with gelatin and c) spherification with alginate.

Other food additives that are widely used in *nouvelle cuisine* are modified starches that are obtained after treating native starch with physical, enzymatic and chemical procedures to improve their properties, particularly as thickener, so it has interesting applications in some ‘modern’ and innovative desserts. Moreover, carboxymethylcellulose is a cellulose derivative that acts as thickener, emulsifier or stabilizer in many recipes and also some enzymes such as transglutaminase can be used to cross-link food proteins and mend novel textures.

However, as these ingredients and techniques are very new, there are no studies investigating their effects on bioactive compounds stability and bioaccessibility.

4.2. Bioavailability of bioactive ingredients

In order to assimilate the nutritional and bioactive compounds present in food, humans submit them to a rough process to degrade complex molecules into simpler compounds easier to be absorbed. This process is called digestion and it starts in the mouth where, besides chewing and other physical processes, saliva including amylase facilitates mainly polysaccharide degradation. Although a lingual lipase initiates fat digestion in the oral cavity, it is almost undigested until it reaches the stomach. When the chewed food (called bolus) is transported there through peristaltic actions, protein digestion is initiated with the action of gastric juices containing pepsin and hydrochloric acid. Some lipid-degrading enzymes are also active at this step [148]. Afterwards, the obtained chyme enters the small intestine where, with the help of the pancreas and gallbladder, lipids are finally digested. In the duodenum, the action of pancreatic lipases, bile acids, lecithin and other molecules leads to the conversion of dietary fat into oily drops and large vesicles that reduced their size until smaller micelles, giving rise to a particular emulsion where only those micelles with optimal dimensions are incorporated into the cell membranes. These micelles are called DMM (dietary mixed micelles) (Figure 13). When the chyme is fully digested, most of the nutrients are absorbed through small intestine enterocytes. Some minerals and water can be also reabsorbed in the large intestine and the non-digested products such as fibers can be utilized by colonic microbiota. The fermentation products could be also reabsorbed in the colon and waste material is excreted as feces [149].

However, the effect of these processes on the fungal bioactive compounds is still not well documented [150]. Ergosterol might undergo similar transformations than cholesterol or phytosterols because its chemical structure resembles them. All sterols are incorporated into the DMMs during digestion and they lose their esterified tails before entering in the enterocytes [151]. In fact, the claimed hypocholesterolemic activity for fungal sterols is partially due to their ability to displace cholesterol from these DMMs [148]. Enterocytes display in their luminal membranes specific transporters that recognize them and transfer them into the endoplasmic reticulum. Several works suggested that the Niemann-Pick C1-like 1 protein is necessary for cholesterol intake and it showed high affinity for this molecule, so non-cholesterol sterols, such as ergosterol and other fungal sterols (as well as plant sterols) are incorporated in very low amounts compared to cholesterol (approx. 2 – 5% vs. 60%) [152, 153].

Moreover, ergosterol hypocholesterolemic activity might go beyond cholesterol displacement, since some works reported its ability to modulate genes related to the cholesterol metabolism in enterocyte-like cells (Caco 2 cells) [49, 154, 155]. However, the low absorption level might prevent the molecule to reach other potential targets and to exert the other beneficial properties before mentioned (but assayed *in vitro*) placing ergosterol bioavailability as a critical point to improve to bypass the intestinal barrier. In this sense, some microemulsion and microencapsulation protocols were evaluated using, for instance, liposomes systems, in order to enhance ergosterol solubility and bioavailability. Yi et al. (2012) [156] succeeded to load *Flammulina velutipes* sterols in a microemulsion system, enhancing its oral bioavailability and antitumoral properties and later on, they also obtained promising results using liposomal encapsulation [157]. Recently, Rudke et al. (2019) [158] also optimized ergosterol microencapsulation of ergosterol and *A. bisporus* extracts using whey protein and chitosan by complex coacervation. Nevertheless, it is still unclear whether plant or fungal sterols should be absorbed in higher concentrations to exert their biological activities since when taken in large amounts they may induce disorders such as sitosterolemia [159].

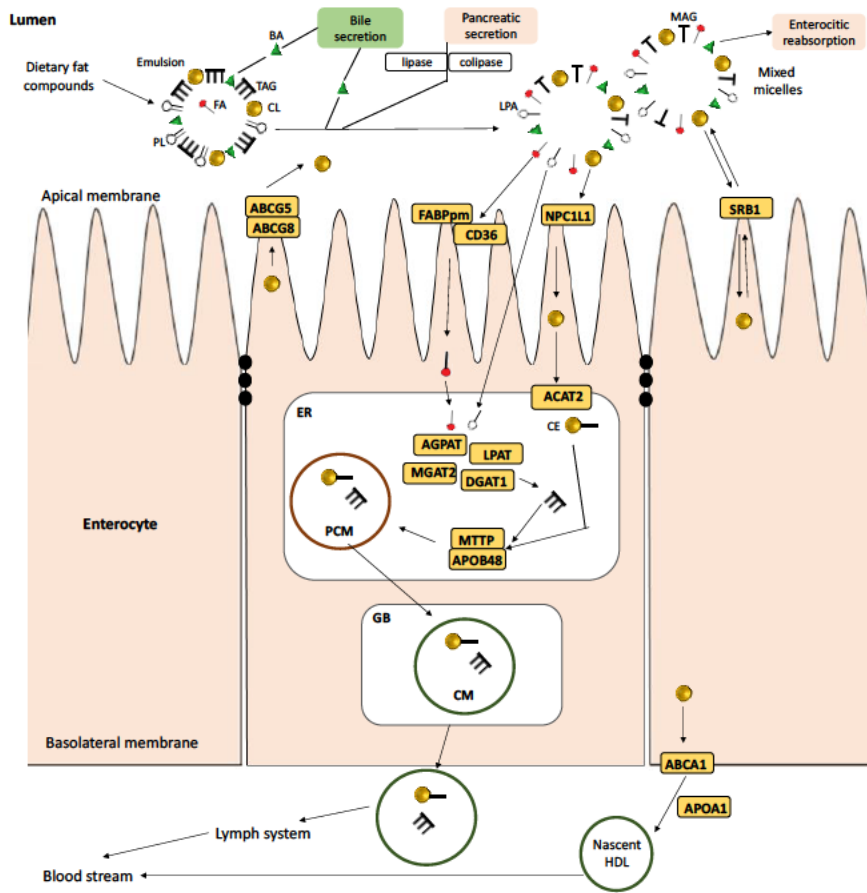


Figure 13. Fat digestion and absorption pathway. PL: phospholipids, FA: fatty acids, BA: bile acids, TAG: triacylglycerols, CL: cholesterol, LPA: lysophosphatidic acid, MAG: monoacylglycerols, LPA: lysophosphatidic acid, CE: cholesterol esters, ABCG5: ATP-binding cassette subfamily G member 5, ABCG8: ATP-binding cassette subfamily G member 8, ABCA1: ATP-binding cassette subfamily A member 1, ACAT2: Acetyl-CoA Acetyltransferase 2, FABPpm: Plasma membrane fatty acid-binding protein, NPC1L1: Niemann-Pick C1-like protein, SRB1: Scavenger receptor class B member 1, AGPAT: 1-acylglycerol-3-phosphate O-acyltransferase, LPAT: lysophosphatidate acyltransferase, MGAT2: monoacylglycerol acyltransferase-2, DGAT1: diacylglycerol O-acyltransferase 1, MTTP: microsomal triglyceride transfer protein large subunit, APOB48: apolipoprotein B-48, APOA1: apolipoprotein A-1, ER: endoplasmic reticle, GB: Golgi body, PCM: pre-chylomicron; CM: chylomicron.

From Gil-Ramirez et al. (2018) [148].

Most of the fungal polysaccharides usually survive human digestion as they are mainly dietary fibers and they are either fermented by enteric microbiome or excreted in the feces. However, the information describing the precise mechanism, or the amount/type of fermented fibers generated from edible mushrooms is scarce. Nevertheless, a few publications suggested that the water soluble β -D-glucans might pass through the enteric barrier via M cells or dendritic cells to reach Peyer's patches [160-162]. Moreover, Gil-Ramirez et al. (2017) [161] observed that water-soluble β -D-glucans were not only bioaccessible but also absorbable according to an *in vitro* digestion model followed by a transport assay using a Caco2 cells line model. Even so, further studies are necessary using *in vivo* animal and human models to a better understanding of mushroom glucans and polysaccharides digestion and bioavailability.

Similarly, only a few studies investigated the bioavailability of mushroom phenolic compounds. Heleno et al. (2015) [163] submitted *Hericium* spp. extracts to an *in vitro* digestion model and observed that most of the phenolic compounds were bioaccessible. However, antioxidant activity was reduced in the digestates, suggesting that digestion process could affect to the structure and therefore the properties of the antioxidant molecules. On the other hand, Soler-Rivas et al. (2009) [164] observed using an *in vitro* digestion model that digestion enhanced the free radical scavenging capacity of *L. edodes* antioxidants that were mostly phenolic compounds. Its antioxidant activity was reduced during mastication but gastric and intestinal digestion increased this activity suggesting that enzymatic activity and pH changes could release or generate new structures with higher antioxidant activity. Moreover, absorption studies using Caco2 cells showed that these antioxidants were potentially bioavailable.

4.3. Effects of *L. edodes* bioactive ingredients on gut microbiome composition

Nowadays it is totally assumed by the scientific community that gut microbiota plays a key role in human health status. Therefore, a large number of food products and food ingredients are being tested because of their ability to act as

prebiotic modulating gut microbiome towards healthier profiles. Microbial communities exert important activities such as biosynthesis of several vitamins for the host, fermentation of polysaccharides and oligosaccharides that human cannot degrade and release of short-chain fatty acids that modulate many important processes. For instance, they seemed to enhance metal cations absorption, eliminate carcinomas, reduce pH (reducing infections), stimulate the GALT, reduce the chances of enterocytic neoplasia and influence cholesterol biosynthesis pathway among others [165]. Microbiome can also promote tolerance of the immune system to commensal microorganisms and rejection to pathogenic strains. Mushrooms contain high amounts of prebiotic polysaccharides such as β - and α -D-glucans or chitins, so several works have been recently developed to study their effect as microbiome-modulators. Some reports described their ability to regulate gut microbiome, for instance, decreasing the relative abundance of genera that were usually correlated with obese or hyperglycemic subjects or stimulating immune system through microbiota [166, 167]. However, further research is required to fully elucidate the involved mechanisms.

Only a few works investigated the effect of *L. edodes* fibers on animal microbiomes. When hypercholesterolemic rats were administrated the mushroom fruiting bodies, the gut microbiome showed a wider number of microbial species compared to control group. The microbiome profile was characterized by increased abundance of *Bacteroides* and *Clostridium* species. It could be concluded that gut microbiota modulation managed dyslipidemia since total cholesterol and LDL cholesterol levels were decreased and HDL cholesterol concentration was increased, finding correlations in the mushroom group [168]. Other work using shiitake mushrooms was published reporting that a combination of aronia, red ginseng, nattokinase (an enzyme produced by *Bacillus natto*) and shiitake mushroom prevented gut microbiome modulation caused by diabetes in rats. The combination also stimulated insulin secretion and reduced insulin resistance. However, the fact that all the ingredients were administered together, it was hard to validate the contribution of the shiitake compounds in these results [169].

5. Application of advanced technologies to obtain compounds from edible mushrooms

Conventional extraction technologies using water or organic solvents, enzymes or physical treatments have been used for decades to recover different mushroom constituents. However, they are being replaced by more environmentally-friendly methods such as microwave-, pulsed electric field-, ultrasound-assisted procedures and also pressurized extractions using food grade supercritical or subcritical fluids [170].

With the latter technologies, high temperatures and pressures can be reached overcoming several disadvantages noticed in conventional extraction methods e.g. long extraction times, low selectivities and the need of toxic or non-food grade solvents [171]. These benefits and their respect for the principles of the ‘green chemistry’ place these technologies on a very relevant position within the food industry.

5.1. Application of supercritical fluids to edible mushrooms

Several studies described the use of supercritical fluids in mushrooms for the extraction of particular fractions, separation of specific compounds, biofuel generation, lipids removal and improvement of extracts quality by inactivating oxidative enzymes. Supercritical CO₂ (scCO₂) was mainly used as solvent because of its excellent physicochemical properties, including high density and diffusivity and low viscosity [171], although, depending on the compounds of interest, other co-solvents were also utilized.

5.1.1. Methods using scCO₂ for the extraction of bioactive compounds

The CO₂ compressed until its supercritical status is frequently used as food-grade extractive fluid because of its non-polar behavior allowing a highly selective extraction of lipophilic compounds leaving no trace after depressurization. Although mushrooms are considered low-fat foods, scCO₂ was used to obtain specific bioactive molecules including fungal sterols, fatty acids and particular fractions with biological activities due to still unidentified compounds [172, 173].

L. edodes and other different mushrooms species were submitted to scCO₂ extractions under a wide range of pressures, temperatures, times and flow rates (Table 4). The largest pressure tested was 60 MPa that was also pumped at the highest tested flow rate (80 kg/h) [174]. However, the pressures described in many studies were between 9-30 MPa, with scCO₂ flows between 0.1 and 3.6 kg/h. Moreover, extractions were also frequently carried out at 40 °C, temperature above the CO₂ critical point (31.1 °C) but low enough to avoid degradation of thermolabile compounds. The effect of higher temperatures such as 60, 85 and 100 °C was also evaluated [174-177]. Many procedures set 3 h as extraction time, although other times were tested adjusting them from 0.2 to 6 h depending on the nature of the extracted compounds or the plant design. Obtained yields varied depending on the raw material and the extraction parameters, but they were often between 0.5 and 4% (w/w) since scCO₂ extractions achieved high levels of selectivity and mushrooms contained low lipid concentrations. Nevertheless, Li et al. (2016) [174] managed to extract 47 g/100 g of *G. lucidum* spores albeit using extreme operating conditions.

The different tested parameters were adjusted to obtain high concentrations of particular bioactive lipids. Ergosterol and other fungal sterols present in mushrooms in lower concentrations such as fungisterol, ergosta-5,7-dienol and ergosta-7,22-dienol were successfully recovered from *A. bisporus* (40 °C, 30 MPa) obtaining extracts with more than 50% (w/w) ergosterol [137]. Similar fractions were also extracted from *L. edodes* [178, 179]. These ergosterol-enriched extracts obtained using SFE from *A. bisporus* showed hypocholesterolemic activity by impairing cholesterol absorption (*in vitro*) when added to lipid matrices such as lard following a similar mechanism than phytosterols and they also modulated mRNA expression of cholesterol-related genes [48, 49]. Other compounds such as specific saturated/unsaturated fatty acids were recovered from mushrooms using SFE. Abdullah et al. (1994) [180] found palmitic and stearic acids in the extracts from *A. bisporus* (50 °C, 28.1 MPa) while Kitzberger et al. (2007) [178] and Mazzutti et al. (2012) [176] extracted palmitic and linoleic acids from *L. edodes* (40 °C, 15 MPa) and *A. blazei* (40, 50, 60 °C, 10, 20, 30 MPa), respectively.

Table 4. Utilized parameters and obtained yields when bioactive compounds were extracted from *L. edodes* and other edible/medicinal mushrooms using supercritical CO₂. (*)Values under the critical point were also tested.

Mushroom species	Pressure (MPa)	Temperature (° C)	Time (h)	Flow (kg/h)	Yield (%w/w)	Main extracted compounds	Ref.
<i>Lentinula edodes</i>	15-30	30-50 *	3	0.2	0.6	Palmitic and linoleic acid, ergosterol	[178]
	15-20	30-50 *	3	0.2-0.4	0.5-1.0	Palmitic and linoleic acid, ergosterol	[179]
	18	40	3	3.4	-	Ergosterol and derivatives	[182]
<i>Agaricus bisporus</i>	28.1	40	0.2	0.2	-	Ergosterol	[183]
	9-30	40	3	3.4	0.6-0.8	Ergosterol and derivatives	[137]
	9-30	40	3	2.4	0.6-0.8	-	[184]
	30	40	3	3.4	-	Ergosterol and derivatives	[48]
	18	40	3	3.4	-	Ergosterol and derivatives	[49]
<i>Agaricus bisporus/Agaricus silvicola</i>	28.1	50	0.2	0.2	-	Palmitic, stearic and benzoic acid	[180]
<i>Agaricus blazei</i>	34.5	60	2	0.1	-	-	[175]
	10-30	40.2-60.2	3.5	0.7	0.5-1.2	Linoleic and palmitic acid	[176]
<i>Ganoderma lucidum</i>	25-30	40-50	3.5	0.2	1.4-2.1	-	[185]
	5-60	32-85	0.5-6	5-80	37	Ganoderic acids	[174]
	10-20	40-50	2	-	1.2	Ganoderic acids	[186]
<i>Ganoderma applanatum</i>	10-30	40	-	0.1	-	Secondary metabolites with DNA-binding activity	[187]
<i>Boletus edulis</i>	10-30	40	1-3	-	0.6-2.4	Linoleic, oleic, palmitic acid	[181]
<i>Boletus luteus</i>	-	35.8-45.1	6	-	1.1-6.1	Oleoresins	[188]
<i>Pleurotus ostreatus</i>	40	100	-	-	0.4	Phenolic compounds	[177]
<i>Antrodia camphorata</i>	34.5	60	2	0.1	-	-	[189]
<i>Hericiium erinaceus</i>	20	40	3	1.5	0.5	-	[190]

High levels of linoleic acid were also detected in *B. edulis* extracts [181] while ganoderic acids (bioactive triterpenoids with the ability to suppress cholangiocarcinoma cell migration) were obtained from *G. lucidum* [174]. Recently, Mahmoud et al. (2016) [177] extracted polyphenols from *P. ostreatus* (100 °C, 40 MPa) generating antioxidant nanoparticles with *in vitro* antitumoral activities after an encapsulation process.

Although extraction of bioactive fractions from mushrooms was the usual main objective for scCO₂ extractions, sometimes they were also used as first step to remove non-polar substances facilitating the posterior extraction of other interesting compounds. Ker et al. (2005) [175] pretreated the mycelium of *A. blazei* with scCO₂ (60 °C, 34.5 MPa) to eliminate oil-soluble substances and minimize the interference during the extraction of antioxidant polysaccharides. The same procedure was carried out by Chen et al. (2007) [189] on *Antrodia camphorata* mycelium.

5.1.2. Methods using scCO₂ plus co-solvents for the extraction of bioactive compounds

Many relevant compounds present low solubility in scCO₂, thus, extraction yields might be very low if only this solvent is used. In these cases, low percentages of another solvent (called co-solvent or modifier) with polarity index could be added to overcome the problem and increase their extractability [191]. When SFE is carried out on edible mushrooms, mostly ethanol was utilized as co-solvent, albeit less polar solvents were also tested such as dichloromethane or ethyl acetate [178]. In all the studies, ethanol percentages did not exceed 15% (w/w) (Table 5).

Addition of limited co-solvent amounts led to an increase of global yields, suggesting that a relevant content of more polar compounds was present in all studied mushroom species. Hsu et al. (2001) [186] enhanced the extraction yield from 1.2 to 1.7% (w/w) in SFE of *G. lucidum* (40 °C, 10 MPa) by adding ethanol as co-solvent. Gil-Ramirez et al. (2013) [137] increased the extraction yield up to 2.1 % (w/w) in *A. bisporus* using 10% ethanol, while Kitzberger et al. (2007) [178] and Mazzutti et al. (2012) [176] reached yields up to 3.8% (w/w) with 15% ethanol in *L. edodes* and 10% in *A. blazei*, respectively. Moreover, a 4-fold increase was also achieved by adding

15% ethanol compared with pure scCO₂ extractions when *H. erinaceus* was used as extracting material [190].

Table 5. Utilized parameters and obtained yields when bioactive compounds were extracted from *L. edodes* and other edible/medicinal mushrooms using supercritical CO₂ and co-solvents. DCM: dichloromethane; EA: ethylacetate. (*)Values under the critical point were also tested.

Mushroom species	Pressure (MPa)	Temperature (° C)	Time (h)	Flow (kg/h)	Co-solvent	Yield (% w/w)	Main extracted compounds	Ref.
<i>Lentinula edodes</i>	15-30	30-50 *	3	0.2	Ethanol (5-15%); DCM (10-20%); EA (15%)	0.5-3.8	-	[178]
	20	40	3	0.2	Ethanol (5-15%)	1.0-3.8	-	[179]
<i>Agaricus bisporus</i>	9-30	40	3	3.4	Ethanol (10%)	1.5-2.1	Ergosterol and derivatives	[137]
	9-30	40	3	2.4	Ethanol (10%)	1.6-2.1	-	[192]
	9-30	40	3	-	Ethanol (10%)	-	Ergosterol and derivatives, immune-modulatory compounds	[184]
<i>Agaricus blazei</i>	10-30	40.2-60.2	3.5	0.7	Ethanol (2.5-10%)	1.5-3.8	Linoleic and palmitic acid, phenolic compounds	[176]
<i>Ganoderma lucidum</i>	10-20	40-50	2	-	Ethanol (-)	1.7-2.1	Ganoderic acids	[186]
<i>Pleurotus ostreatus</i>	15-25	40-60	1.5	18	Ethanol (-)	-	Phenolic compounds and ergothioneine	[113]
<i>Hericium erinaceus</i>	20	40	3	1.5	Ethanol (10%)	1.0-2.0	-	[190]

The addition of ethanol to scCO₂ extractions of *L. edodes* generated fractions with higher amounts of phenolic compounds and higher antioxidant capacity. Their antimicrobial activity (against *E. coli*, *S. aureus*, *B. cereus*, *Micrococcus luteus* and *Candida albicans*) remained similar to the one noticed for the extracts obtained using

only scCO₂ [178]. However, the fractions obtained from *A. blazei* by adding 10% ethanol showed lower antimicrobial activity than without the co-solvent, indicating that in this mushroom, non-polar substances were involved in the inhibition of microbial growth [176]. Moreover, SFEs carried out on *P. ostreatus* pointed out that the optimal conditions to obtain extracts with high concentrations of antioxidant compounds such as ergothioneine and polyphenols were 48 °C and 21 MPa using ethanol as co-solvent [113]. SFE extracts obtained from *A. bisporus* combining scCO₂ with 10% ethanol showed a remarkable immunomodulatory effect. The extracts obtained at 9, 18 and 30 MPa reduced the IL-10 levels in THP-1/M differentiated macrophages (activated with LPS) leaving the values of other cytokines (IL-6, IL-1 β and TNF- α). However, ergosterol was not the responsible for this activity since extracts obtained with only scCO₂ contained higher sterol concentrations and showed no immunomodulatory activities [184].

5.1.3. Methods using scCO₂ and subcritical water for the extraction of bioactive compounds

Supercritical CO₂ was also utilized as secondary solvent in a complex mixture used to extract β -D-glucans from *G. lucidum*. Pressurized hot water and sub/supercritical CO₂ (133-175 °C, 5-10 MPa) were used to achieve acidification of the media improving the hydrolysis reactions that enhanced β -D-glucan extraction [193]. Indeed, when CO₂ was added above its critical point, the extraction yield of β -D-glucans was dramatically improved (from 58 to 73 % w/w at 155 °C and 8.5 MPa).

5.1.4. Supercritical fluid chromatography to separate fungal molecules

In some cases, supercritical fluid chromatography (SFC) showed advantages compared to high performance liquid chromatography (HPLC) such as higher separation efficiency, shorter analysis time and lower consumption of organic solvents [194].

Supercritical fluids, particularly scCO₂, were used as mobile phases for the identification and quantification of fungal metabolites in complex extracts. For instance, ergosterol was used as fungal biomarker in food products to detect mould

contamination [183]. Flour or moldy breads were submitted to SFC at 50 °C and 30.2 MPa on a 250 x 4.6 mm stainless steel column of Spherisorb Amino (3 µm) using scCO₂ mixed with 10% methanol. SFC was also carried out to separate different epimers such as pair of 25 *R/S*-ergostanes and tetracyclic triterpenoids with interesting bioactive properties (antitumoral, anti-inflammatory, immunomodulatory, antiviral, hepatoprotective and antihypertensive) from *Anrodia camphorata* [195-197].

5.2. Application of subcritical solvents to edible mushrooms

Bioactive compounds can also be extracted by pressurizing the solvent below its critical point and then, it is considered as a subcritical solvent. This approach is known as pressurized liquid extraction (PLE) or accelerated solvent extraction (ASE). Still high pressures are used offering the possibility to heat the solvent above its boiling point, keeping its liquid state and conferring it different properties than those at room temperature and atmospheric pressure. Several polar molecules were fractionated from mushrooms using subcritical water extractions (SWE), although other solvents were also tested.

5.2.1. Methods using subcritical water for extraction of bioactive compounds

Subcritical water (SW) is a highly versatile solvent because temperature can modulate its dielectric constant and therefore its polarity, allowing extractions of not only polar compounds but also non-polar molecules when temperature is above 100 °C. In addition, SW has advantages over other solvents, for instance it is non-toxic so it does not need to be removed for some food applications, and it shows low disposal costs [198].

Several mushroom species were submitted to SWE to generate different types of interesting fractions (Table 6). A wide range of pressures (4-25.3 MPa), temperatures (25-350 °C), extraction times (5-300 min) or extraction cycles were evaluated obtaining yields up to 90.3% (w/w). These values were significantly higher than those obtained with SFE, especially with pure scCO₂. SWE was mostly carried out to recover bioactive polysaccharides such as β-D-glucans.

Smiderle et al. (2017) [143] submitted some mushrooms species to SWE to define the optimal conditions to extract mainly polysaccharides. The pressures were maintained between 10.2-11.7 MPa while temperature (50-180 °C) and extraction time (5-30 min) were the experimental variables in a full-factor experimental design. They noticed that temperature was the main variable influencing polysaccharide extraction. The highest polysaccharide yields were reached at 180 °C for *P. ostreatus* (23-37% w/w) and *G. lucidum* (8-12%). Higher extraction temperatures were also tested for *G. lucidum* and, although some authors indicated that 200 °C (10 MPa, 130 min) were the optimal conditions to recover β -D-glucans (reaching 0.44 mg β -D-glucans / 100 g dw) [199], other publications observed a decrease in β -D-glucans solubility and other water soluble organic compounds above 158 °C [193]. Askin et al. (2010) [199] noticed this loss of solubility as well but at temperatures higher than 200 °C.

Other tested extraction variables were the use of extraction cycles (1 – 15), the extraction time of each cycle (1 – 5 min) and the ratio sample/sand mixed in the extraction cell (1:4; 1:8 and 1:16 mushroom:sand). Within the same temperature, a sample extracted for longer time and shorter number of cycles showed similar extraction yields to those using a shorter extraction time but more cycles number. No significant polysaccharide yield increase was observed from 5 to 15 cycles and apparently, all the extractable polysaccharides were already obtained after 5 cycles. The cell was not saturated with ratios 1:4, thus, higher amount of mushroom powder resulted in higher yield [76, 192, 208]. Moreover, the possibility of carrying out sequential instead of individual extraction was also evaluated and it was noticed that individual extractions yielded larger amount of polysaccharides from *L. edodes* than using a progressive increase of temperatures [76].

In several publications, SWE (particularly using 150 or 200 °C) was reported as a more effective method for polysaccharide extraction than conventional extraction methods (operating at 120 °C for 20 min, or 100 °C for 30-60 min) [76]. In other studies, the yield was not significantly different and, in the case of *B. edulis*, a conventional method was reported to extract more polysaccharides than SFE due to

lower selectivity [208]. In the latter mushroom, both methods extracted similar amounts of β -D-glucans. Similar results were also noticed for *G. lucidum* [204].

Table 6 (Part A). Parameters, solvents and conditions utilized and yields obtained when bioactive compounds were extracted from edible or medicinal mushrooms using subcritical fluids.

Subcritical fluid	Mushroom species	Pressure (MPa)	Temperature (°C)	Time (min)	Yield (% w/w)	Main extracted compounds	Ref.
Water	<i>Lentinula edodes</i>	2.5-25.3	28	10-80	27.9-90.3	Polysaccharides	[200]
		10.7	25-200	25	20.1-82.3	β -D-glucans and other polysaccharides	[76]
	<i>Agaricus bisporus</i>	10.7	25-200	25	26.7-78.3	β -D-glucans and other polysaccharides	[76]
		10.7	25-200	25	18.5-74.9	β -D-glucans and HMGCR inhibitors	[192]
	<i>Ganoderma lucidum</i>	5-10	25-360	5-60	7.5-78.1	Ganoderic alcohols and ganoderic acids	[201]
		10.2-11.7	50-180	5-30	0.2-11.9	β -D-glucans and other polysaccharides	[143]
		10	100-300	5-130	3.0-78.1	Polysaccharides	[199]
		4	100-190	300	-	Polysaccharides	[202]
		4	120-180	60	-	β -D-glucans and other polysaccharides	[203]
		5	135-175	31-171	-	β -D-glucans	[193]
		10.7	25-200	5-75	29.3-36.9	β -D-glucans and chito-oligosaccharides	[204]
		10.2-11.7	50-180	5-30	2.5-30.3	β -D-glucans and other polysaccharides	[143]
	<i>Pleurotus ostreatus</i>	10.7	25-200	25	15.4-78.6	β -D-glucans and other polysaccharides	[76]

Table 6 (Part B). Parameters, solvents and conditions utilized and yields obtained when bioactive compounds were extracted from edible or medicinal mushrooms using subcritical fluids.

Subcritical fluid	Mushroom species	Pressure (MPa)	Temperature (°C)	Time (min)	Yield (% w/w)	Main extracted compounds	Ref.
Water	<i>Pleurotus cornucopiae</i> var. <i>citrinopileatus</i>	5	50-300	10-60	-	Phenolic compounds and β -D-glucans	[205]
		5	100-230	5-55	-	Polysaccharides	[142]
	<i>Grifola frondosa</i>	>4	121-150	30-60	-	Phenolic compounds and β -D-glucans	[206]
	<i>Inonotus obliquus</i>	5	50-300	10-60	-	Phenolic compounds	[207]
	<i>Boletus edulis</i>	10.7	25-200	5-75	20.7-79.7	β -D-glucans and chito-oligosaccharides	[208]
Ethanol	<i>Agaricus bisporus</i>	10.7	50-100	1-75	4.3-27.3	Ergosterol and derivatives	[137]
	<i>Agaricus bisporus</i>	10.7	50	25	-	Ergosterol and derivatives	[48]
	<i>Pleurotus eryngii</i>	10.7	25-200	25	1.8-15.3	-	[209]
Water + ethanol	<i>Agaricus bisporus</i>	10.7	25-200	25	6.2-33.3	β -D-glucans and HMGCR inhibitors	[192]
Petroleum ether	<i>Ganoderma lucidum</i> / <i>Ganoderma sinense</i>	10.3	160	10	-	Ergosterol and fatty acids	[210]

Other studies carried out using *A. bisporus*, *P. ostreatus* and *L. edodes* indicated that extracts obtained with SWE at high temperatures (200 °C) showed similar β -D-glucans content and similar bile acids binding-capacities than those obtained with the conventional method, except for *L. edodes* SWE extract, that showed a significantly higher hypocholesterolemic activity [76]. These extractions, carried out at 200 °C, led to a 3-, 4- and 5-fold increase in extraction yields compared with extractions at 25 °C (5 cycles of 5 min and 10.3 MPa). However, the fractions obtained from those mushrooms when using mild temperatures (25 – 50 °C) were also biologically interesting, showing other hypocholesterolemic properties and

immunomodulatory activities. Extracts obtained at 25 °C (10.7 MPa after 5 extraction cycles of 5 min each) inhibited the HMGCR (*in vitro*) [192, 211]. The authors suggested that water-soluble but thermolabile polysaccharides (α -, β -D-glucans and fucomannogalactans in *L. edodes* and α -, β -D-glucans and mannogalactans in *P. ostreatus*) might be involved in the observed inhibitory activity [65, 161]. SWE fractions obtained at 50 °C (10.7 MPa for 5 cycles of 5 min extraction) from *A. bisporus*, *L. edodes*, *B. edulis*, *P. ostreatus* and *G. lucidum* containing α - and β -D-glucans and chitins (mainly at 200 °C) but also proteins (mainly in those at 50 °C) showed immunomodulatory activities. They modulated the release of cytokines such as TNF- α , IL-6, IL-1 β (pro-inflammatory cytokines) and IL-10 (anti-inflammatory interleukin) when applied at subtoxic concentrations to LPS-activated THP-1/M cells. Extracts from *L. edodes* obtained at 50 °C reduced the secretion of all tested cytokines. Similar extracts but obtained from *G. lucidum*, *A. bisporus*, *P. ostreatus* and *B. edulis* reduced IL-10 levels, while extracts from *G. lucidum* and *A. bisporus* obtained at 200 °C stimulated IL-10 production [184].

Other SWEs were carried out to obtain antioxidant compounds. Yang et al. (2013) [142] compared the antioxidant capacity of *G. frondosa* polysaccharides extracted by SWE or conventional extraction (100-230 °C, 5-55 min) using a response surface methodology. The optimal extraction conditions were 210 °C and 43.6 min of extraction time and water-to-raw material ratio of 26:1, yielding 2 fold more polysaccharides (25.1%) than conventional extractions. Moreover, SWE extracted polysaccharides showed higher reducing power and DPPH \cdot scavenging activities than hot water extracted fractions. *G. frondosa* apparently contained other antioxidants besides polysaccharides such as phenolic compounds [206]. The antioxidant properties of the obtained extracts increased with temperature and time and correlated with the total phenols content reaching a maximum value when extracted at 150 °C for 60 min. β -D-glucans were also detected, but they were mainly extracted at 140 °C within extraction times of 30 min.

The antioxidant capacity and total phenols content of SWE fractions obtained from *I. obliquus* were also directly related with temperature, although a decrease was observed with temperatures above 300 °C [207]. Similar relevance of the extraction

temperature was noticed for *Pleurotus cornucopiae* var. *citrinopileatus*. The SWE fractions obtained at 200, 250 and 300 °C showed the highest values of β -D-glucans, total phenols and antioxidant capacity, respectively [205].

Selenium-enriched mushrooms (*A. bisporus*) were also submitted to SWE to obtain specific bioactive extracts with hypocholesterolemic and antioxidant activities. Selenium is essential for the proper functioning of antioxidant enzymes such as glutathione peroxidases (preventing LDL oxidation) and it might enhance the effect of HMGCR inhibitors. Results indicated that only those fractions obtained at 25 °C (at 10.7 MPa for 25 min) showed HMGCR inhibitory activity. The amount of selenium in the obtained fractions increased with the temperature up to 150 °C. In this fraction, it was present in the same concentration than the raw mushrooms and further extractions up to 200 °C yielded fractions with lower amounts [212].

5.2.2. Methods using other subcritical solvents for extraction of bioactive compounds

Other solvents such as ethanol and petroleum ether were pressurized under subcritical conditions to extract particularly hydrophobic compounds (fungal sterols and other lipids), although different solvent combinations were also examined.

The use of subcritical mixtures of ethanol:water (1:1) was compared to SW for the extraction of compounds with HMGCR inhibitory activity from *A. bisporus*. The solvent combination resulted in a slightly lower amount of compounds at 25 °C. In contrast, the use of higher temperatures (50, 100 °C) produced extracts with similar yields that did not inhibit the enzyme, probably because of the thermal instability of the bioactive compounds [192].

SWE and subcritical ethanol extraction (SEE) were also carried out on *P. eryngii* to obtain inhibitors of the pancreatic lipase [209]. In both extractions, the increase of temperatures led to higher yields. However, when using water the extracted material was 3 to 20 fold higher than when using ethanol. Unfortunately, none of the generated extracts showed interesting inhibitory activities when using an *in vitro* digestion model.

Fungal sterols were extracted with subcritical ethanol at 50 and 100 °C from *A. bisporus* and compared with SFE extractions [137]. SEE extracted higher amounts of compounds than scCO₂ (up to 27.3 % w/w), however, their sterol content was significantly lower (approx. 5% in SEE vs 50% in SFE), indicating that SFE was a more selective method to obtain ergosterol-enriched fractions. Moreover, when the ability of both extracts was tested as cholesterol displacers from the dietary mixed micelles using an *in vitro* digestion model, SEE extracts seemed to be less effective than SFE extracts, probably due to their lower sterols contents [48].

Ergosterol together with fatty acids (palmitic, linoleic, oleic, stearic acids, etc.) was also extracted with subcritical petroleum ether at 10.3 MPa and 160 °C for 10 min from *G. lucidum* and *Ganoderma sinense*. However, since this solvent is not food-grade, the extractions were carried out as part of a novel method to determine and compare the content of those compounds in the different varieties of the two *Ganoderma* species [210].

5.3. Microwave-assisted extraction (MAE)

Besides pressurized technologies, other novel environmentally-friendly extraction methods such as MAE and UAE were tested to obtain bioactive compounds from mushrooms. They all share the advantages of SFE and PLE compared to conventional extractions i.e. solvents are non-toxic and they can be used in lower amounts, extraction times are shorter and solvent removal steps are not required. However, when compared with pressurized methods, the selection of one or another method may be influenced by particular pros and cons.

In MAE, microwaves are used as non-contact, effective and selective heat sources that accelerate energy transfer, start-up and response to heating control. This technology reduces extraction times, thermal gradient and equipment size compared with conventional heating. No large amounts of solvents are needed since it might also be carried out without solvent [213]. Several MAE procedures were described to extract bioactive compounds from mushrooms, mainly ergosterol, phenolic compounds and polysaccharides.

Smiderle et al. (2017) [143] compared the ability of MAE and SWE to obtain polysaccharides (particularly β -D-glucans) from *P. ostreatus* and *G. lucidum* using a full-factorial experimental design (response surface methodology). In both cases, extraction yields were more influenced by temperature than by extraction time. In fact, the latter parameter did not affect SWE and only slightly did to MAE. The polysaccharides extracted using both technologies were similar (differences were only quantitative) and the included mainly β - and α -D-glucans and heteropolysaccharides according to NMR analysis. The highest polysaccharide yields were obtained at the highest tested temperature (180 °C) with both methods. Nevertheless, one of the SWE advantages was the use of pressure that was significantly higher (10.2-11.7 MPa) than in MAE (max. 1.5 MPa) and that improved extraction efficiency, probably because it helped introducing the solvent deeper inside the sample matrix. SWE carried out using *P. ostreatus* showed higher yields than MAE, however, no significant differences were noticed for *G. lucidum* extractions. Another SWE advantage was operational since the extract could be directly recovered in a vessel, whereas MAE samples required centrifugation steps to separate the extract. The SWE disadvantage respect to MAE was the lower reproducibility, particularly for *P. ostreatus* extractions. The authors concluded that both methods could be used as easy, fast and efficient procedures to extract mushroom polysaccharides.

Some polysaccharides with *in vitro* antioxidant and antitumoral activities from *Armillaria luteovirens* were also extracted using MAE (following a Box-Behnken method design), obtaining approx. 8.4% yield at the optimal conditions [214]. Unfortunately, this mushroom was not submitted to PLE to make a comparison, but they were close to those obtained with *G. lucidum* when using MAE [143]. MAE was also applied to *Terfezia boudieri*, *B. edulis* and *Lactarius volemus* to obtain antioxidant extracts with high levels of total phenols using several methanol concentrations [216]. Similar solvent mixtures such as ethanol/water were also tested on *T. versicolor* [217] yielding 470 mg total phenols/100 g extract with antioxidant properties. Wise to mention was the very short extraction time for optimal conditions that was estimated in both publications as 5 and 3.8 min, respectively. This extraction speed and the fact that SFE fractions from *A. blazei* contained lower phenols contents

(32 mg/g) [176] pointed MAE as a more interesting technology than SFE to obtain phenol-enriched extracts.

Heleno et al. (2016) [217] utilized MAE at short times (3-20 min) and high temperatures (60-210 °C) to recover ergosterol from *A. bisporus* by-products such as low quality fruiting bodies, lower part of the stipe, etc. The ergosterol yields of MAE were similar to those reported by Gil-Ramirez et al. (2013) [137] using SFE although higher than those obtained with SEE, indicating that MAE could also be a suitable technique for extraction of fungal sterols.

5.4. Ultrasound-assisted extraction (UAE)

Several mushroom strains were also submitted to other advanced extraction technologies such as UAE. UAE is used for compounds extraction from biological materials because the ultrasonic waves produced during its application provoke implosions of the generated cavitation bubbles [218]. In mushrooms, these implosions cause a disruption of fungal cell walls accelerating diffusion through their membranes, enhancing mass transfer and extraction yields.

Antioxidant polysaccharides were obtained from *G. lucidum* using UAE and compared with more conventional extraction methods such as hot water or Soxhlet extraction [219]. Apparently, when exposed to ultrasounds for 60 min, 8.1 mg of polysaccharides/g mushroom could be obtained. Those values were higher than the obtained with conventional extraction procedures, but lower than those obtained with SW at 25 °C (154.8 mg/g mushroom). These values were even lower when compared with SWE extractions carried out at 200 °C, where almost 260.3 mg/g were recorded [204]. Mushroom polysaccharides were also extracted from *A. bisporus* low-quality products (stipes and caps that are misshaped or do not meet the specifications set by retailers) using UAE. The application of ultrasounds enhanced the polysaccharide extraction yields being the highest (4.7%) achieved with an extraction time of 15 min, maximum amplitude of 100 µm with 1 h of precipitation in 80% ethanol [220]. Polysaccharide extractions from the same mushroom species carried out using SWE reached higher yields (10.4%) [76], however, high temperatures (200 °C) were needed and thus, it might be detrimental to extract specific thermolabile compounds. Other

mushroom species were submitted to UAE to obtain polysaccharides with biological properties such as *L. edodes* [221], *B. edulis* [222] or *Trametes orientalis* [223] and the extraction conditions were slightly different. The highest polysaccharide yield extracted from *T. orientalis* (7.49%) was obtained with 109.8 W, 40.2 °C and 42.2 min). For the extraction of antioxidant polysaccharides from *B. edulis*, different UAE procedures (ultrasonic clearer, static probe and pulsed counter-current probe extractions) were tested being the counter-current probe ultrasonic extraction the technique that registered the maximum yields (8.21%). Yet, these values were still lower than those obtained by SWE (from 8.8 to 20.9%), reached at temperatures from 100 to 200 °C [208]. Polysaccharide-enriched extracts with anti-hepatitis B activities (*in vitro*) were also generated after submission of *L. edodes* fruiting bodies to UAE [221]. At the optimal conditions (45 °C, 21 min, 290 W), a yield of 9.75% was obtained representing a 1.62-fold increase compared with conventional hot water extraction. However, it was lower than when using SWE (at 200 °C) where a 21% yield was noticed [76]. In order to improve the polysaccharide yields of ultrasonic extractions, Chen et al. (2010) [224] combined them with MAE to optimize a novel procedure named ultrasonic/microwave assisted extraction (UMAE). The optimal conditions for UMAE were 90 W of microwave power, 50 W of ultrasonic power together with an ultrasonic frequency of 40 kHz, a solid/water ratio of 1:20 (w/v) and an extraction time of 19 min. Under these conditions, fractions from *I. obliquus* were obtained with a 3.25% yield containing 73.16% of antitumoral polysaccharides.

Other molecules such as ergosterol were extracted from *A. bisporus* with UAE but using ethanol as solvent. At the optimal conditions, the obtained sterol extraction yielded 671.5 mg sterol/100 g mushroom (dw), values higher than Soxhlet extractions with interesting reductions in the extraction time (15 min and 4 h, respectively) [225]. SWE carried out using the same mushroom yielded 618 mg sterol/100 g mushroom when 200 °C were selected and SFE extracted 525 mg/100 g with scCO₂ and up to 495 mg/100g when mixed with 10% ethanol as cosolvent [137]. Nevertheless, extractions with pressurized fluids yielded fractions with higher ergosterol content (up to almost 60% w/w) pointing SFE and SWE as more selective technologies to obtain fungal sterols.

Furthermore, less frequently extracted compounds such as melanins were also obtained by UAE as a healthy colorant from *A. auricula-judae* [226]. However, no reports are available until now on the use of pressurized fluid technologies for the extraction of this molecule.

5.5. Other advanced technologies

Other interesting advanced technologies could be used to extract interesting compounds such as instant controlled pressure drop (DIC) and pulsed-electric fields (PeF)-assisted extractions although only in PeF extractions mushrooms were used as starting material.

DIC submits raw materials to saturated steam during a short time and immediately applied a drastic pressure drop towards vacuum leading to the vaporization of volatile compounds, instant cooling of the material and expansion of cell walls, enhancing mass transfer and desired compounds recovery. This technology was used to extract volatile and antioxidant compounds from plants [227].

PeF are very short (micro- to milliseconds), high-voltage electricity pulses that, when impacting on food placed between specific electrodes inside a chamber, enhance compounds extraction by modifying cells permeability [228]. PeF technology was used to extract mushroom compounds because hyphae are easily electropermeabilizable enabling high recoveries of intracellular components. However, only a few studies were published using mushrooms as starting material.

PeF (12.4 to 38.4 kV/cm) were applied to *A. bisporus* suspensions to extract several compounds. The highest yields were obtained when increasing the field intensity and temperature, e.g. 7.9 mg/g polysaccharide, 1.6 mg/g phenols and 2.7 mg/g proteins were obtained when submitted to electric pulses of 38.4 kV/cm field intensity for 272 μ s at 85 °C [229]. Combination of PeF (0.8-1.3 kV/cm) with pressure (0.5 MPa) was also tested [230] to extract the same compounds from *A. bisporus*. However, the developed method was not effective extracting phenolic compounds, requiring a subsequent ethanol extraction step. Submission of *A. bisporus* fruiting bodies to SWE at 100 °C extracted 14.1 mg polysaccharides/g mushroom. An increase in temperature (up to 200 °C) resulted in concentrations of 103.7 mg/g vs. the

83.5 mg/g obtained with the conventional extraction method (120 °C, 20 min) [76]. These results suggested that PeF could be interesting only to obtain specific polysaccharides, for instance, those with anticoagulant activities extracted from *A. auricula-judae* suspensions [231].

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Objectives / Objetivos



Objectives

According to previous works, edible mushrooms, particularly shiitake mushrooms (*Lentinula edodes*), are an interesting source of bioactive ingredients with different biological properties such as hypocholesterolemic, antioxidant, antitumoral, immunomodulatory, anti-inflammatory, antihypertensive, prebiotic-activity, etc., and therefore, they could be used to design novel functional foods. However, many of those activities were evaluated with *in vitro* assays or animal studies while human trials are scarce. Lab testing provides highly valuable information as well as cell cultures or *in vivo* testing but, when a functional food is intended to be launched to the market, human testing is essential to get the confirmation of its beneficial effect and the approval of the health claim by authorities.

Therefore, the objective of this work was to compare different advanced extraction technologies, considered as environmentally friendly, with other more conventional procedures to obtain fractions from *L. edodes* with different bioactivities. The biological properties of the fungal extracts were tested *in vitro* and with animal models and, the most promising extracts, in a clinical trial.

In order to achieve this main objective, the work plan was divided in a few milestones organized depending on the chemical nature of the bioactive molecule and separated in chapters in this thesis. Depending on the type of compound, a few extraction technologies were adjusted to obtain specific fractions and afterwards, their biological activities were tested:

1. Production of extracts containing fungal sterols and ergocalciferol and evaluation of their hypocholesterolemic properties:

- *Development of an extraction method using a pilot-scale supercritical CO₂ extraction plant to obtain ergosterol-enriched extracts.

- *Transformation of ergosterol-enriched extracts into vitamin D₂-enriched extracts using UV-irradiation.

- *Adjustment of the developed extraction method to a larger scale supercritical CO₂ extraction plant to obtain large amounts of ergosterol-enriched extracts.

- *Microemulsification of ergosterol and ergosterol-enriched extracts.
- *Study of the ability of ergosterol and ergosterol-enriched extracts (with/without β -D-glucan supplementation) to displace cholesterol from dietary mixed micelles using an *in vitro* digestion model.
- *Demonstration of the hypocholesterolemic activity of ergosterol, ergosterol-enriched extracts and a β -D-glucan-enriched extract using mice models.

2. Production of extracts containing eritadenine and evaluation of its hypocholesterolemic properties:

- *Screening of edible mushrooms to select the optimal source of eritadenine.
- *Defining the optimal developmental stage and tissue to extract eritadenine from *L. edodes* fruiting bodies.
- *Selection of the more adequate method to extract and quantify eritadenine.
- *Evaluation of the effect of traditional and modern culinary processing on eritadenine stability and bioaccessibility.
- *Demonstration of the biosafety, bioavailability and hypocholesterolemic activity of eritadenine-enriched extracts using a rat model.

3. Production of β -D-glucan-enriched extracts and evaluation of their biological activities:

- *Development of a microwave-assisted extraction method to obtain β -D-glucan-enriched extracts.
- *Optimization of the methods to obtain β -D-glucan-enriched extracts by combining ultrasound-assisted and/or subcritical-water extractions with/without pre-treatment with supercritical CO₂.
- *Development of a pilot scale method using a solid/liquid extraction unit coupled to pressure-driven crossflow filtration membranes to obtain large amounts of β -D-glucan-enriched extracts.
- *Determination of the total β -D-glucans, (1 \rightarrow 3)- β -D-glucans and (1 \rightarrow 3),(1 \rightarrow 6)- β -D-glucans of the extracts by colorimetric and fluorimetric methods and verification by GC-MS, HPSEC and NMR.
- *Isolation of specific glucans from the β -D-glucan-enriched extracts.

*Study of the hypocholesterolemic, antioxidant, immunomodulatory and antitumoral activities using *in vitro* tests and cell cultures of the most interesting β -D-glucan-enriched extracts and purified glucans.

4. Design of a scalable sequential procedure to optimize the extraction of several bioactive fractions from the same batch of shiitake fruiting bodies or by-products:

* Establishment of a specific sequence interconnecting extraction methods to obtain several bioactive fractions simultaneously from a single *L. edodes* batch.

*Determination of the fractionated bioactive compounds (β -D-glucans, ergosterol, eritadenine, lenthionine, phenolic compounds, chitins, hypotensive peptides).

*Evaluation of their antioxidant, hypocholesterolemic and antihypertensive activities using *in vitro* tests.

5. Demonstration of the biological activities of a designed functional food in a human trial:

* Preparation of a bioactive extracts mixture in sufficient concentrations to carry out a clinical trial and mixing with specific food matrices.

*Study of the hypocholesterolemic and immunomodulatory properties of the functional food in a double-blind and parallel clinical trial.

*Evaluation of the microbiome-modulatory properties of the administrated functional food by qPCR and bioinformatics tools.

Objetivos

De acuerdo con estudios previos, las setas comestibles, particularmente las setas shiitake (*Lentinula edodes*), constituyen una interesante fuente de ingredientes bioactivos con diferentes propiedades biológicas tales como hipocolesterolémica, antioxidante, antitumoral, inmunomodulatoria, anti-inflamatoria, antihipertensiva, prebiótica, etc. Por lo tanto, pueden ser empleadas para diseñar nuevos alimentos funcionales. Sin embargo, muchas de estas actividades han sido evaluadas en ensayos *in vitro* o utilizando modelos animales, si bien los estudios en humanos son escasos. Los análisis a escala de laboratorio proporcionan información muy valiosa, al igual que los test en cultivos celulares o *in vivo* pero, cuando se pretende lanzar un alimento funcional al mercado, los ensayos en humanos son esenciales para confirmar su efecto beneficioso y lograr la aprobación de sus declaraciones de propiedades saludables por parte de las autoridades.

Por ello, el objetivo de este trabajo fue comparar diferentes tecnologías de extracción avanzadas, consideradas como respetuosas con el medioambiente, con otros procedimientos más convencionales para la obtención de fracciones a partir de *L. edodes* con diferentes bioactividades. Las propiedades biológicas de los extractos fúngicos fueron evaluadas *in vitro* y con modelos animales, y los extractos más prometedores, en un ensayo clínico.

Para alcanzar este objetivo principal, el plan de trabajo se dividió en diversos objetivos secundarios en función de la naturaleza química de la molécula bioactiva y estos objetivos se agruparon en los distintos capítulos de esta tesis. Dependiendo del tipo de compuesto, varias tecnologías de extracción fueron ajustadas para obtener fracciones específicas y, posteriormente, sus actividades biológicas fueron evaluadas:

1. Producción de extractos que contienen esteroides fúngicos y ergocalciferol y evaluación de sus propiedades hipocolesterolémicas:

*Desarrollo de un método de extracción utilizando una planta de extracción con CO₂ supercrítico a escala piloto para obtener extractos enriquecidos en ergosterol.

*Transformación de los extractos enriquecidos en ergosterol en extractos enriquecidos en vitamina D₂ utilizando irradiación con UV.

*Ajuste del método de extracción desarrollado a una planta de extracción con CO₂ supercrítico a mayor escala para obtener grandes cantidades de extractos enriquecidos en ergosterol.

*Microemulsión de ergosterol y de los extractos enriquecidos en ergosterol.

*Estudio de la habilidad del ergosterol y los extractos enriquecidos en ergosterol (con o sin suplementar con β -D-glucanos) para desplazar el colesterol de las micelas mixtas de la dieta utilizando un modelo de digestión *in vitro*.

*Demostración de la actividad hipocolesterolémica del ergosterol, los extractos enriquecidos en ergosterol y en β -D-glucanos utilizando modelos de ratón.

2. Producción de extractos que contienen eritadenina y evaluación de sus propiedades hipocolesterolémicas:

*Análisis de diferentes especies de setas comestibles para seleccionar la fuente óptima de eritadenina.

*Determinación de la etapa de desarrollo y el tejido óptimo para la extracción de eritadenina de cuerpos fructíferos de *L. edodes*.

*Selección del método más adecuado para la extracción y cuantificación de eritadenina.

*Evaluación del efecto del procesado culinario tradicional y moderno en la estabilidad y bioaccesibilidad de la eritadenina.

*Demostración de la bioseguridad, biodisponibilidad y actividad hipocolesterolémica de extractos enriquecidos en eritadenina utilizando un modelo de ratón.

3. Producción de extractos enriquecidos en β -D-glucanos y evaluación de sus actividades biológicas:

*Desarrollo de un método de extracción asistida por microondas para la obtención de extractos enriquecidos en β -D-glucanos.

*Optimización de métodos de obtención de extractos enriquecidos en β -D-glucanos mediante la combinación de extracciones asistidas por ultrasonidos y/o con agua subcrítica con/sin un pretratamiento con CO₂ supercrítico.

*Desarrollo de un método a escala piloto utilizando una unidad de extracción sólido/líquido acoplada a un sistema de filtración por membranas de flujo tangencial impulsado a presión para la obtención de grandes cantidades de extractos enriquecidos en β -D-glucanos.

*Determinación de los β -glucanos totales, (1 \rightarrow 3)- β -D-glucanos y (1 \rightarrow 3),(1 \rightarrow 6)- β -D-glucanos de los extractos utilizando métodos colorimétricos y fluorimétricos y verificación mediante GC-MS, HPSEC y NMR.

*Aislamiento de glucanos específicos a partir de los extractos ricos en β -D-glucanos.

*Estudio de la actividad hipocolesterolémica, antioxidante, inmunomodulatoria y antitumoral de los extractos enriquecidos en β -D-glucanos y de los glucanos más interesantes, utilizando tests *in vitro* y cultivos celulares.

4. Diseño de un procedimiento escalable y secuencial para optimizar la extracción de varias fracciones bioactivas a partir del mismo lote de cuerpos fructíferos de shiitake o subproductos:

*Establecimiento de una secuencia específica interconectando métodos de extracción para obtener varias fracciones bioactivas de forma simultánea a partir de un solo lote de *L. edodes*.

*Determinación de los compuestos bioactivos fraccionados (β -D-glucanos, ergosterol, eritadenina, lentinina, compuestos fenólicos, quitinas, péptidos hipotensivos).

*Evaluación de su actividad antioxidante, hipocolesterolémica y antihipertensiva utilizando test *in vitro*.

5. Demostración de la actividad biológica de un alimento funcional diseñado en un ensayo clínico en humanos:

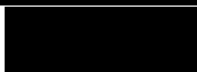
*Preparación de una mezcla de extractos bioactivos en concentraciones suficientes para llevar a cabo un ensayo clínico y mezclado con matrices alimentarias específicas.

*Estudio de las propiedades hipocolesterolémicas e inmunomodulatorias del alimento funcional en un ensayo clínico doble-ciego y paralelo.

*Evaluación de las propiedades moduladoras del microbioma del alimento funcional administrado mediante qPCR y herramientas bioinformáticas.

Chapter 1

Production of extracts containing
ergocalciferol and fungal sterols



Preface

Fungal sterols were reported as bioactive compounds because they showed several biological activities such as antioxidant, antitumoral, anti-inflammatory and hypocholesterolemic, being this last one studied in more detail. The specific sterol level depended on the mushroom strain but they all included ergosterol as main compound since it is the major constituent of the hyphal membrane. Moreover, ergosterol is the vitamin D₂ (ergocalciferol) precursor and, although this vitamin is present in low amounts in mushrooms cultivated in darkness, its photoconversion can be stimulated by UV-irradiation of the fruiting bodies. Vitamin D was also able to influence cholesterol homeostasis as low levels were correlated with high total cholesterol levels and high hyperlipidemia development risk. In addition, it was also involved in many vital metabolic pathways e.g. bone and calcium metabolism, etc.

L. edodes can be used as source of fungal sterols and extracts with high levels of ergosterol might be obtained using SFE. Later on, these extracts can be transformed into ergocalciferol-enriched extracts by UV-irradiation. In the work entitled *Vitamin D-enriched extracts obtained from shiitake mushrooms (Lentinula edodes) by supercritical fluid extraction and UV-irradiation*, supercritical CO₂ extractions were carried out testing different operational parameters and following a specific experimental design to produce extracts with high ergosterol yields. Afterwards, these SFE extracts were exposed to UV-C and UV-B lights as well as to a light with wide spectrum (200-700 nm) under several conditions and ergosterol was transformed into several intermediate compounds generating fungal extracts with interesting vitamin D₂ concentrations. The transformation was > 100 folds more effective than when fruiting bodies were directly irradiated.

Later on, in the work entitled *In vitro and in vivo testing of the hypocholesterolemic activity of ergosterol- and β -D-glucan-enriched extracts obtained from shiitake mushrooms (Lentinula edodes)*, supercritical CO₂ extractions were performed but, in this case, a plant with higher dimensions was utilized to produce larger amounts of ergosterol-enriched fractions to carry out *in vitro* and *in vivo* analysis. Parameters were adjusted using those defined in the smaller SFE plant as a basis and fractions were collected in two independent separators. The SFE extract

containing higher ergosterol concentrations was tested as cholesterol displacer from DMMs using an *in vitro* digestion model. The extract was also microemulsified to improve its bioavailability and mixed with a β -D-glucan-enriched mixture (BGE) also containing eritadenine (that will be fully described in chapter 4) to investigate whether these processes might enhance/impair the hypocholesterolemic activity of the SFE extract noticed *in vitro*.

Moreover, both ergosterol and ergosterol-enriched extract were administrated to mice simultaneously with a hypercholesterolemic diet to confirm their *in vitro* hypocholesterolemic activity. However, no effective lowering of cholesterol in animal serum was noticed. Thus, since the BGE mixture also showed interesting ability to displace cholesterol *in vitro*, another *in vivo* test was carried out using hypercholesterolemic mice. When the mixture was administrated as a palliative extract, significant cholesterol reduction was noticed. Therefore, no further studies were planned with fungal sterols and efforts were focused on the study of eritadenine and β -D-glucan-enriched extracts (chapters 2 and 3).

Manuscript 1

Vitamin D-enriched extracts obtained from shiitake mushrooms (*Lentinula edodes*) by supercritical fluid extraction and UV-irradiation

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Abstract

The combination of supercritical fluid extraction followed by UV-irradiation resulted in an interesting strategy to obtain ergosterol- and vitamin D₂-enriched extracts from *Lentinula edodes* to design novel functional foods. The extractions carried out following a specific experimental design pointed out that extraction yields and ergosterol concentrations were more influenced by pressure than by the extraction temperature, although high temperatures (55–75 °C) might induce transformation of ergosta-7,22-dienol and fungisterol into ergosterol. After extraction, the ergosterol-enriched extracts should be dissolved in methanol or ethanol and irradiated (25 °C, 4 cm) to partially transform ergosterol into vitamin D₂. Irradiation at 365 nm was less effective than at 254 nm. The fastest transformation was obtained using an UV lamp covering the complete UV spectrum for a maximum of 1h. However, this lamp also induced vitamin D₄ formation although in lower amounts than vitamin D₂ or lumisterol₂ while with irradiation at 254 nm most of ergosterol was transformed into vitamin D₂.

Industrial relevance: This work describes a new method to enhance the vitamin D content of hypocholesterolemic extracts obtained from edible mushrooms such as *Lentinula edodes* (shiitake mushrooms). The generated extracts might be used to design novel functional foods by simple adjustment of the amount of ergosterol that wants to be transformed into vitamin D. Firstly, the mushrooms are submitted to supercritical fluid extraction (a process that can be up scaled since nowadays it is widely utilized for coffee decaffeination) to obtain ergosterol-enriched fractions. Then, they might be dissolved in GRAS solvents such as ethanol and irradiated with UV light to generate vitamin D. Irradiation of an extract obtained from the mushroom seems more effective than direct fruiting bodies irradiation, since > 100 folds vitamin D can be generated.

Introduction

Edible mushrooms contain many different hypocholesterolemic compounds such as β -D-glucans, fungal sterols, specific strain-dependent compounds, etc. that could be extracted and utilized to design bioactive ingredients for novel functional foods. Several studies indicated that these molecules might modulate cholesterol homeostasis by inhibiting the endogenous cholesterol biosynthesis and impairing exogenous cholesterol absorption [1,2]. However, there might be many other indirect mechanisms involved in the regulation of serum cholesterol levels since, for instance, mushroom extracts containing eritadenine, an inhibitor of the S-adenosyl-L-homocysteine hydrolase (a key enzyme in the hepatic phospholipid metabolism), lowered total cholesterol levels *in vivo* [3]. Vitamin D might also indirectly affect cholesterol levels (besides the other already known calcium or parathyroid-related metabolic pathways) since low vitamin D status was associated with high total cholesterol levels and an increased risk of developing hyperlipidemia [4,5].

The different vitamin D structures are synthesized from provitamins D that are temporarily transformed by UV irradiation into previtamins D. The latter intermediate can generate tachysterols, lumisterols or vitamins D depending on light and temperature [6]. The main vitamins D found in foods are ergocalciferol (or vitamin D₂), cholecalciferol (or vitamin D₃) and 22,23-dihydroergocalciferol (or vitamin D₄). The vitamin D form found in blood serum is generated in liver by cholecalciferol hydroxylation originating 25-hydroxycholecalciferol. However, some reports preferred to name it as 25-hydroxyvitamin D since ergocalciferol can also be bioavailable yielding 25-hydroxyergocalciferol and improving the levels of total 25-hydroxyvitamins D [7].

Mushrooms contain mainly vitamin D₂ (with traces of the other vitamins D [7]) but their levels are largely dependent on environmental conditions. Those picked from the woods usually showed higher levels than indoor cultivated mushrooms. However, they all contain ergosterol (and other derivative sterols) because it is a constitutive compound in fungal hyphae as well as an ergocalciferol (vitamin D₂) precursor. Transformation of ergosterol into vitamin D₂ takes place during the development of fruiting bodies exposed to light, however, it can be reproduced *in*

vitro by exposing their caps or gills to UV-light [8, 9]. *Pleurotus* spp. fruiting bodies increased their vitamin D₂ content from almost 0 to approx. 60 or 200 µg/g depending on the analysed strain after 2 h UV-B irradiation [10]. Lower irradiation times yielded lower transformation but still 30 min exposure increased vitamin D₂ levels in *A. bisporus* and *L. edodes* up to respectively 119.21 and 59.89µg/g [9]. Exposure of gills facing UV-A source induced 4.4 fold more transformation of ergosterol into vitamin D₂ than cap exposure [8] being UV-B irradiation more effective than UV-A or UV-C. Recent works indicated that ergosterol enriched fractions obtained from mushrooms using supercritical fluid extractions (SFE) were able to lower cholesterol levels in hypercholesterolemic mice [11], however, they might be even more effective if ergosterol is partly transformed into vitamin D₂ as noticed on hypercholesterolemic patients treated with statins after vitamin D supplementation [12]. Therefore, in this work a new method to extract ergosterol using SFE and convert it into vitamin D₂ is described. The UV irradiation was carried out once the extract was generated and not on the mushroom fruiting body, as has traditionally been done.

Materials and Methods

Biological material and reagents

Powdered *Lentinula edodes* S. (Berkeley) fruiting bodies were purchased from Glucanfeed S.L. (La Rioja, Spain) and stored in darkness at -20 °C until further use. Obtained powder showed a particle size lower than 0.5 mm and moisture content lower than 5%.

Solvents as hexane (95%), chloroform (HPLC grade) and methanol (HPLC grade) were obtained from LAB-SCAN (Gliwice, Poland) and absolute ethanol and sea sand from Panreac (Barcelona, Spain). Potassium hydroxide, ascorbic acid and BHT (2,6-Di-tert-butyl-p-cresol) as well as hexadecane, ergosterol (95%), ergocalciferol (99%) (vitamin D₂) and cholecalciferol (98%) (vitamin D₃) were purchased from Sigma-Aldrich Química (Madrid, Spain). The CO₂ (99,99% purity) was supplied by Air-Liquid España, S.A. (Madrid, Spain). All other reagents and solvents were used of analytical grade.

Supercritical fluid extractions (SFE)

Supercritical fluid extractions with CO₂ were carried out in a plant (Thar Technology, Pittsburgh, PA, USA, model SF2000) comprising a 2 L cylinder extraction cell and two different separators (S1 and S2) of 0.5 L capacity each with independent control of temperature and pressure. The extraction vessel had a ratio of 5.5 height/ diameter. A detailed explanation of the experimental device can be found elsewhere [13]. The extraction cell was filled with shiitake powder (253 g) and washed sea sand (1100 g) in a ratio of 1:1 (v/v). The temperature of separators 1 and 2 was set to 40 °C for all the experimental assays and the pressure of S1 and S2 was maintained at 60 bar for all the extractions. The CO₂ flow was set at 3.6 kg/h and during the total extraction time (3 h) it was recirculated. The compounds extracted in both separators were washed with ethanol and immediately submitted to concentration until dryness on a rotary vacuum evaporator.

Table 1. Central composite design (2² + star design) of supercritical extractions from *Lentinula edodes*.

Run	Variables	
	Temperature (°C)	Pressure (MPa)
1	33.8	22.5
2	70.0	10.0
3	55.0	22.5
4	55.0	22.5
5	76.2	22.5
6	40.0	10.0
7	40.0	35.0
8	55.0	22.5
9	55.0	40.2
10	70.0	35.0
11	55.0	4.8

The dried extracts were stored at $-20\text{ }^{\circ}\text{C}$ until further analysis. Extracted dry matter content was measured to calculate the extraction yields. Extraction yields were expressed as percentage of dry matter (in grams) obtained from 100 g of dry raw material utilized for extraction.

In order to optimize the extraction method to obtain sterol-enriched fractions, parameters such as extraction pressure and extraction temperature were tested following a central composite design (2^2 + star design). All the experiments (see Table 1) were fully randomized to provide protection against the effect of lurking variables. Values for extraction temperature and pressure ranged respectively from 33.8 to 76.2 $^{\circ}\text{C}$ and 4.8 to 40.2 MPa, with star points corresponding to 33.8 and 76.2 $^{\circ}\text{C}$ in the case of temperature, and 4.8 and 40.2 bar in the case of pressure.

UV-irradiation of SFE extracts

Sterol-enriched extracts obtained by SFE and lyophilized shiitake mushrooms were submitted to UV-irradiation using two different lamps: a Höhensonne 100 quartz lamp from Original Hanau (Hanau, Germany) that emits UV radiation covering a wide light spectrum (200–700 nm) since it coupled an IR rod (WS-UV); and a VL-4.LC lamp from Vilber Lourmat (Eberhardzell, Germany) that can irradiate specifically at 254 (UV-C) or 365 nm (UV-A).

Powdered *L. edodes* fruiting bodies (50 mg) were mixed with 3 mL of different solvents (water, methanol and ethanol) in 2.5 cm diam. \times 8 cm height cylindrical vessels, and exposed uncovered to the radiation under vigorously shaking at a distance of 26 (Höhensonne lamp) or 14 (VL-4 lamp) cm for different incubation times (0, 15, 30, 60 and 120 min). Other distances to the lamp were also tested and therefore, the vials were placed at 4, 14 and 24 cm far from the UV source. Similarly, the fractions obtained after SFE extractions (12 mg) were dissolved and treated as previously mentioned for the powdered fruiting bodies.

GC-FID-MS analysis

Fungal sterols from both shiitake mushrooms and SFE fractions (irradiated and non-irradiated) were extracted following the procedure described by Gil-Ramirez

et al. (2013) [14]. The unsaponified fractions obtained (6 mg/mL) were injected into an Agilent19091S-433 capillary column (30 m ×0.25 mm ID and 0.25µm phase thickness). The column was connected to a 7890A System gas chromatograph (Agilent Technologies, USA) including a G4513A auto injector and a 5975C triple-Axis mass spectrometer detector. The injector and detector conditions as well as the column temperature program were those described by Gil-Ramirez et al. (2013) [14]. Ergosterol was used as standard to validate the GC method, using hexadecane (10% v/v) as internal standard.

GC-MS database identified the obtained peaks in concordance with previous studies [8, 14, 15]. The major detected sterols were ergosterol (ergosta-5,7,22-trien-3β-ol) (RT = 12.6 min), ergosta-7,22-dienol (RT =12.8 min), ergosta-5,7-dienol (RT = 13.1min) and ergosta-7-enol (fungisterol) (RT = 13.3 min).

HPLC-DAD and HPLC-MS analyses

The unsaponified fractions obtained as previously described from both shiitake mushrooms or irradiated and non-irradiated SFE fractions were injected (20 µL) into a Varian HPLC, model 920-LC Galaxy, with a diode array (PAD) detector. Reverse phase chromatographic separation was performed with a Carotenoid C30 analytical column (250 × 4.6mm, 5µm) from YMC Europe (Dinslaken, Germany). Solvents utilized as mobile phase were 85% methanol (v/v) (A) and ethanol (B). They were mixed following the gradient: 5% B during 2 min, up to 40% B in 5 min and then maintained 15 min, up to 90% B in 5 min and maintained 5 min more. The flow rate was 1 mL/min and the oven temperature 50 °C. The absorbance changes were followed by a UV-VIS DAD and 265 nm was selected for quantification. Vitamin D₂ (R.T. =16.2 min) and vitamin D₃ (R.T. = 16.5 min) were injected as standards obtaining chromatograms similar to Wittig et al. (2013) [6]. Nevertheless, to identify vitamin D-related structures, samples were also injected (using the above described method and column) into an Agilent 1100 series liquid chromatograph equipped with a PDA detector and directly coupled to an ion trap mass spectrometer (Agilent ion trap 6320) via an atmospheric pressure chemical ionization (APCI) interface. The selected parameters and conditions were: positive ionization mode, capillary voltage, −3.5 kV; drying temperature, 350 °C; vaporizer temperature, 400 °C; drying gas flow

rate, 5 L/min; corona current, 4000 nA; nebulizer gas pressure, 60 psi. Full scan was acquired in the range from m/z 50 to 2200.

Samples were injected in duplicate and ergocalciferol (vitamin D₂) was used as standard for the quantitative determination of vitamins D and derivative compounds.

Statistical data analyses

The one-way ANOVA as well as the Durbin-Watson statistic tests was used to determine the statistical significance of the extraction pressure and temperature on the percentage of ergosterol extracted. Significance was set at $P < 0.05$. Calculations were made using StatGraphics Centurion XVII.I (Statpoint Technologies, Inc., Virginia, USA) software.

The rest of experimental data was analysed for statistical significance by one-way ANOVA followed by Tukey's multiple comparison test using Prism GraphPad 5.03 software (GraphPad Software Inc., San Diego, CA, USA).

Results and discussion

SFE extraction of sterols-enriched fractions

Fruiting bodies from *L. edodes* were submitted to supercritical CO₂ extractions without co-solvent since previous results carried out with both *L. edodes* and *A. bisporus* stated that mixtures including 5, 10 or 15% ethanol (v/v) yielded extracts with higher dry matter but lower sterol contents [14, 16, 17]. Moreover, the use of other solvents such as dichloromethane or ethyl acetate did not improve the extracted amounts [16]. Thus, two extraction variables such as pressure and temperature were tested following the previously described experimental design (Table 1) to estimate the optimal combinations.

To study the level of significance of each factor, an analysis of variance (ANOVA) was performed for two selected responses (extraction yield and ergosterol concentration). To be able to describe the effects of the different factors and

interactions in the response, only the significant factors were chosen (95% confidence level).

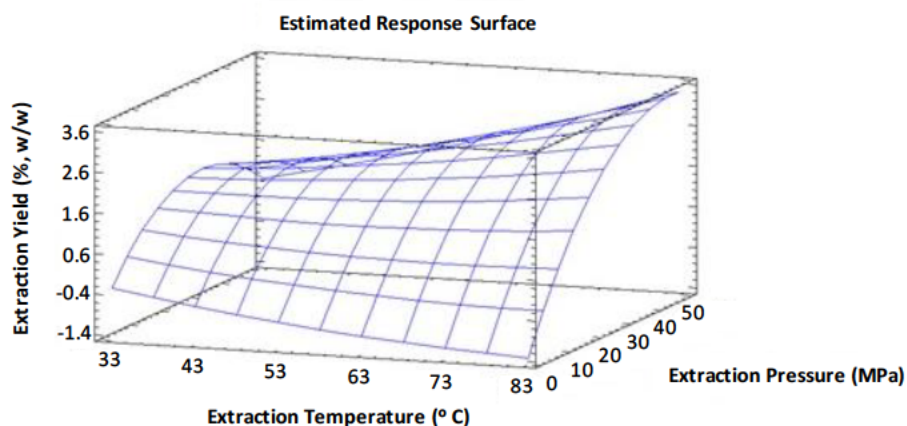
The ANOVA analysis of the extraction yield showed that only the factor extraction pressure had a P -value < 0.05 , indicating that it was significantly different from zero at the 95% confidence level. The Response Surface Plot for the extraction yield (% w/w) (Figure 1a) indicated that the extraction yields were much more influenced by the extraction pressure, being the highest of 3.28%, at the conditions of 35 MPa and 70 °C. These results were in concordance with those of Kitzberger et al. (2009) [17] that reported that high pressures combined with the increase of the operational temperature led to the enhancement of the extraction yield of shiitake oil. However, results obtained with conditions such as 22.5 MPa and 75 °C allowed recovery yields of approx. 2.3% (w/w) being higher than those obtained in previous reports (approx. 1%) where lower temperatures and/or similar or higher pressures were selected [17]. Mazzutti et al. (2012) [18] reported yields of 1.19% when working with *Agaricus brasiliensis* at 30 MPa and 50 °C. Previous results using *A. bisporus* as raw material achieved yields of approx. 0.6% at extraction temperatures of 40 °C regardless of the pressure selected (9, 18 and 30 MPa) [14]. In the case of ergosterol concentration, both the extraction pressure and the extraction temperature had P -values < 0.05 , indicating that they were significantly different from zero at the 95% confidence level. The regression equation fitting to the data was:

$$[\text{Ergosterol}] = 48.4066 - 1.42215 * \text{Temperature} + 0.03441 * \text{Pressure} + 0.01362 * \text{Temperature}^2 - 0.00001 * \text{Temperature} * \text{Pressure} + 0.00011 * \text{Pressure}^2$$

The Response Surface Plot obtained by a graphical representation of the fitted equation (Figure 1b) showed the behavior of the response as a function of the different factors values. An increase in both the extraction temperature and pressure led to an increase in the percentage of ergosterol in the extracts. The experimental condition of 35 MPa and 70 °C provided the highest ergosterol percentage (18% w/w) corresponding to 180 mg/g dw (dry weight). This may be explained by the combined effect of a high CO₂ density (0.83 g/cm³) plus the

vapor pressure at these conditions [19, 20]. The ergosterol content of the obtained fractions ranged from 87 mg/g dw (at 8.5 MPa 55 °C).

a)



b)

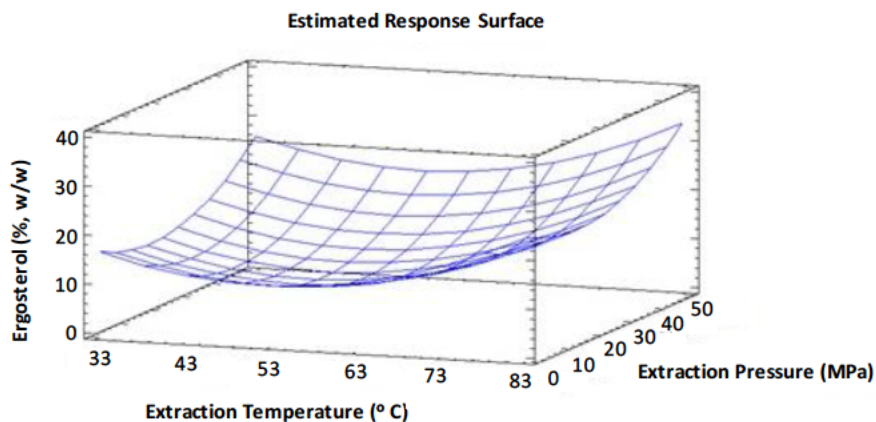


Figure 1. Response Surface Plot for a) extraction yield and b) ergosterol concentration obtained after SFE extractions (expressed in % w/w).

The fraction containing 18% ergosterol (w/w) included other ergosterol derivatives such as fungisterol (2%), ergosta-7,22-dienol (1.7%) and ergosta-5,7-dienol (0.2%). The increase of pressure did not largely influence the extraction of the other fungal sterols, however, an increase of temperature might induce the

transformation of ergosta-7,22-dienol and fungisterol into ergosterol. In extractions obtained at 22.5 MPa, an increase in temperature from 35, 55 to 75 °C led to a rise in the ergosterol levels of respectively 76, 79 and 82% of the total extracted sterols, and to a decrease in ergosta-7,22-dienol levels of respectively 11, 10 and 8% and of 12, 10 and 9% in the case of fungisterol. On contrary, levels of ergosta-5,7-dienol remained constant but they only represented the 1% of the total extracted sterols.

Effect of the UV-lamp utilized for SFE-extracts irradiation

The powdered *L. edodes* strain utilized in this study contained ergosterol but no ergocalciferol (vitamin D₂) (Figure 2). When the mushroom powder was directly exposed to UV-C irradiation (254 nm), ergosterol levels decreased 16% while vitamin D₂ increased. However, when the powder was suspended in methanol, stirred and irradiated, ergosterol levels were reduced 27% and vitamin D₂ levels increased 6.6 folds compared with initial values. These results indicated that irradiation of mushroom powder within a medium was more effective than dry irradiation and even more than direct fruiting body irradiation (if compared with levels previously reported). Fresh *L. edodes* fruiting bodies irradiation for 1 h but at 310 nm (UV-B) induced the formation of 0.004 mg/g vitamin D₂ [21] and 2 h incubation yielded 0.015 mg/g [22]. If they were placed with their gills facing the UV source for 30 min at 20 °C, 0.029 mg/g vitamin D₂ were generated while irradiation of dried fruiting bodies induced up to 0.06 mg/g [9].

Thus, the irradiation of the ergosterol-enriched extracts was carried out using methanol as solvent for two reasons: firstly, because after the above described comparison, the use of an organic solvent was more effective than using none or water to induce vitamin D₂ biosynthesis. The higher solubility of sterol and vitamin D derivatives in organic mixtures might positively influence transformation yields. Secondly, because the oily fractions obtained after SFE were more easily solubilized in organic solvents rather than in aqueous mixtures.

The SFE extract selected for irradiation studies was the one obtained after SFE extraction at 35 MPa and 70 °C due to its higher ergosterol concentration compared to the ones obtained under other experimental conditions. This extract

initially contained 216.9 mg/g fungal sterols where ergosterol represented 82.9% (w/w) of total sterols, ergosta-7,22-dienol and fungisterol were present in lower concentrations (7.7 and 7.6%, respectively), and ergosta-5,7-dienol was also detected but in very low quantities (1.8%) (Table 2).

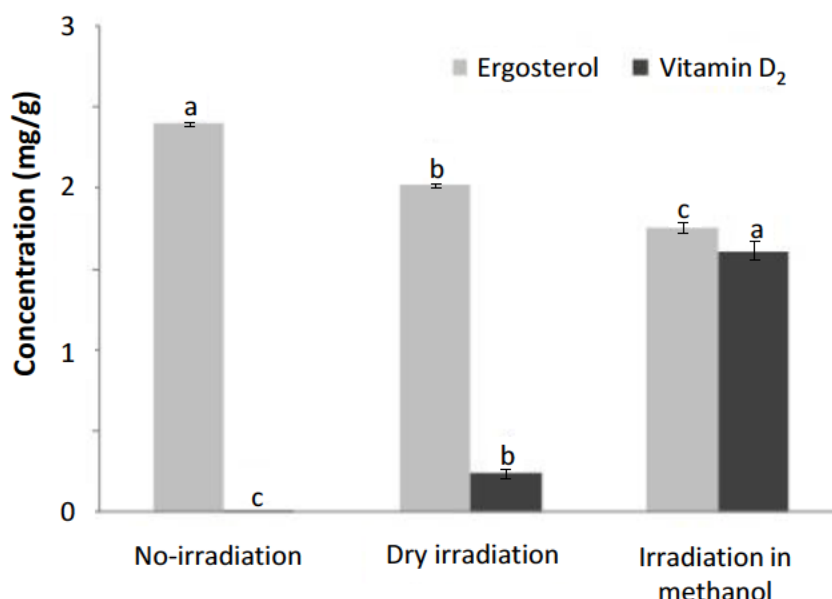


Figure 2. Levels of ergosterol and vitamin D₂ (mg ergosterol or vitamin D₂/g mushroom powder) in powdered Shiitake fruiting bodies non-irradiated and irradiated with UV-C for 1 h at 50 °C. Irradiation was carried out directly on mushroom powder (dry irradiation) or suspended in methanol. ^{a-c} Different letters denote significant differences ($P < 0.05$) between values of the same series.

When the extract was solubilized and irradiated with the lamp able to supply a wide spectrum of UV light (WS-UV), a large reduction in the ergosterol level was noticed until its complete degradation after 2 h incubation (Figure 3). This compound was rapidly transformed into vitamin D₂ since its level was increasing with the irradiation time until 1 h. Afterwards, the UV-light exposure seemed detrimental also for the vitamin, perhaps, the heat generated by the IR rod coupled to the UV-lamp was excessive. IR irradiation was complementary used because heat enhanced UV effect enhancing the transformation of previtamins D into vitamins D rather than into

tachysterols or lumisterols [6]. Levels of the other derivative sterols were also influenced and drastically reduced after 1 h irradiation. They were not expected to be transformed into any other compound due to the UV-light, therefore, their reduction might also suggest a negative influence of the heat generated by the IR rod.

Table 2. Fungal sterols content in the SFE extract irradiated for 2 h using different UV-lamps. n.d. = not detected. ^{a-d} Different letters denote significant differences ($P < 0,05$) between different times of exposure for the same compound and the same lamp.

Lamp	Time (min)	Ergosterol (mg/g)	Ergosta-7,22-dienol (mg/g)	Fungisterol (mg/g)	Ergosta-5,7-dienol (mg/g)	Total sterols (mg/g)
UV-C (254 nm)	0	179.89 ± 11.72 ^a	16.70 ± 0.82 ^a	16.50 ± 0.79 ^b	3.80 ± 0.23 ^a	216.89
	15	174.04 ± 2.00 ^a	8.93 ± 0.55 ^b	17.46 ± 0.23 ^b	3.34 ± 0.60 ^a	203.77
	30	175.82 ± 1.33 ^a	13.71 ± 0.21 ^{ab}	23.90 ± 2.44 ^a	7.13 ± 2.42 ^a	220.57
	60	159.26 ± 1.50 ^a	11.03 ± 0.03 ^b	18.41 ± 1.72 ^{ab}	3.74 ± 0.69 ^a	192.44
	120	88.75 ± 8.82 ^b	10.29 ± 0.53 ^b	22.78 ± 3.45 ^a	3.90 ± 1.04 ^a	125.72
UV-A (365 nm)	0	179.89 ± 11.72 ^a	16.70 ± 0.82 ^a	16.50 ± 0.79 ^a	3.80 ± 0.23 ^a	216.89
	15	181.34 ± 4.18 ^a	11.77 ± 3.13 ^a	24.51 ± 4.65 ^a	4.55 ± 0.77 ^a	222.17
	30	185.51 ± 4.19 ^a	13.26 ± 2.38 ^a	24.08 ± 3.53 ^a	4.68 ± 1.07 ^a	227.53
	60	182.87 ± 2.19 ^a	13.22 ± 2.19 ^a	22.86 ± 3.30 ^a	3.63 ± 0.61 ^a	222.58
	120	175.41 ± 0.24 ^a	11.03 ± 0.86 ^a	21.62 ± 0.94 ^a	3.79 ± 0.54 ^a	211.86
WS-UV	0	179.89 ± 11.72 ^a	16.70 ± 0.82 ^a	16.50 ± 0.79 ^b	3.80 ± 0.23 ^a	216.89
	15	141.74 ± 5.45 ^b	9.85 ± 0.13 ^b	17.95 ± 0.70 ^b	3.31 ± 0.68 ^a	172.85
	30	121.02 ± 6.00 ^b	16.55 ± 2.25 ^a	24.44 ± 1.27 ^a	1.79 ± 0.36 ^a	163.80
	60	74.40 ± 3.60 ^c	9.74 ± 1.71 ^b	17.42 ± 0.22 ^b	1.26 ± 0.04 ^a	102.83
	120	n.d. ^d	n.d. ^c	2.34 ± 0.30 ^c	n.d. ^b	2.34

Irradiation with UV-A (365 nm) induced almost no transformation of ergosterol into vitamin D₂. However, the procedure still yielded more vitamin than described in other reports where the irradiation was carried out directly on mushroom fruiting bodies. SFE extract irradiation for 1 h yielded 0.24 mg/g vitamin D₂ and after 2 h the amount increased up to 1.13 mg/g while the largest amount obtained after 2 h of fruiting bodies UV-A irradiation (with gills facing the source) was 0.023 mg/g [8]. Nevertheless, UV-C irradiation (254 nm) of SFE extracts was more effective than

UV-A because after 2 h UV-C irradiation almost half of the ergosterol content was transformed into vitamin D₂ that doubled its levels. Levels of the other ergosterol derivatives remained constant within the irradiation period independently of the wavelength utilized (Table 2).

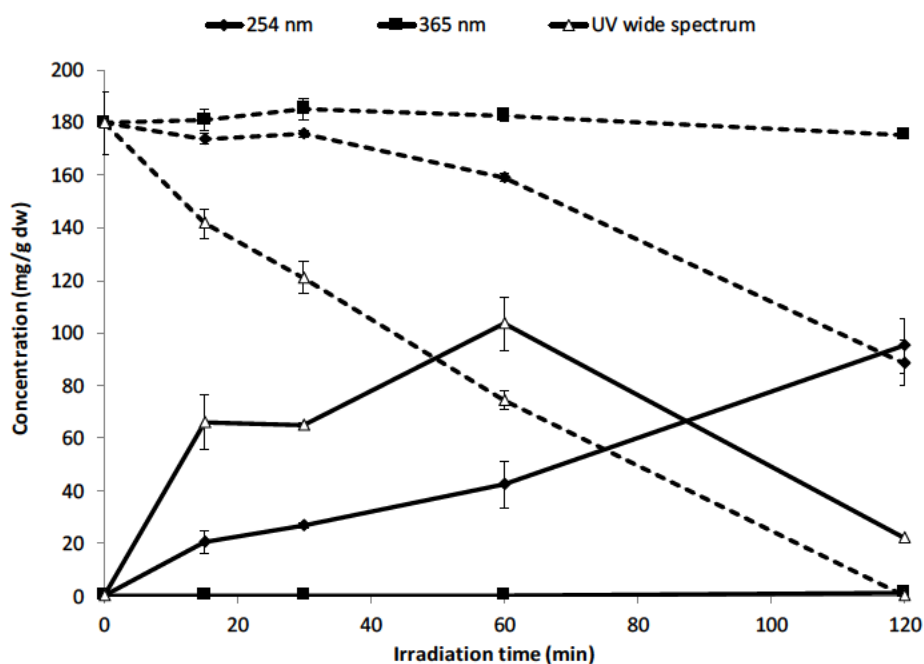


Figure 3. Effect of UV light using different lamps on ergosterol (dashed line) and vitamin D₂ (solid line) levels during 2 h irradiation. Concentration is expressed in mg ergosterol or vitamin D₂/g SFE extract.

Apparently, the UV-lamps utilized also stimulated production of different vitamins D and photoisomers (Table 3). According to HPLC-MS analysis, generated compounds were in concordance with those previously reported by Wittig et al. (2013) [6]. Previtamin D₂ formation could be noticed after 15 and 60 min when irradiated with UV-C and the WS-UV lamps, respectively. But probably, it was further transformed since its levels were reduced further in time concomitantly with an increase of its derivatives such as vitamin D₂, lumisterol₂ and tachysterol₂. Lumisterol₂ was after vitamin D₂ the second previtamin D₂-derivative generated. Its levels were only approx. 3 fold lower than vitamin D₂ after 120 min irradiation in

extracts irradiated with UV-C or after 60 min when irradiation was carried out with WS-UV lamps. The transformation of previtamin D₂ into tachysterol₂ was not stimulated since only low concentrations could be noticed after 60 min WS-UV irradiation. However, vitamin D₄ levels increased up to 60 min when the irradiation was carried out with the WS-UV lamp and was almost not detected with UV-C irradiation. Previtamin D₄, tachysterol₄ and lumisterol₄ were also increased although at lower concentrations than vitamin D₄ during 60 min with WS-UV irradiation.

Table 3 (Part A). Vitamins D and photoisomers detected in the SFE extract during 2 h irradiation using different UV-lamps. Previtamin D₂ (PRE₂), tachysterol₂ (T₂), lumisterol₂ (L₂) and vitamin D₂ (V₂). n.d. = not detected.^{a-c} Different letters denote significant differences ($P < 0.05$) between different times of exposure for the same compound and the same lamp.

Lamp	Time (min)	PRE ₂ (mg/g)	T ₂ (mg/g)	L ₂ (mg/g)	V ₂ (mg/g)
UV-C (254 nm)	0	n.d. ^a	n.d. ^a	n.d. ^a	n.d. ^a
	15	n.d. ^a	n.d. ^a	2.53 ± 0.86 ^a	20.54 ± 4.02 ^b
	30	n.d. ^a	n.d. ^a	9.34 ± 0.00 ^a	26.81 ± 0.97 ^b
	60	0.10 ± 0.15 ^a	n.d. ^a	12.38 ± 3.72 ^a	42.38 ± 8.84 ^b
	120	n.d. ^a	n.d. ^a	32.74 ± 24.58 ^a	95.10 ± 10.23 ^a
UV-A (365 nm)	0	n.d. ^a	n.d. ^a	n.d. ^a	n.d. ^a
	15	n.d. ^a	n.d. ^a	n.d. ^a	n.d. ^a
	30	n.d. ^a	n.d. ^a	n.d. ^a	n.d. ^a
	60	0.41 ± 0.58 ^a	n.d. ^a	n.d. ^a	0.24 ± 0.34 ^a
	120	0.33 ± 0.46 ^a	n.d. ^a	0.68 ± 0.96 ^a	1.13 ± 0.31 ^a
WS-UV	0	n.d. ^c	n.d. ^a	n.d. ^c	n.d. ^c
	15	2.18 ± 0.00 ^a	n.d. ^a	27.78 ± 0.09 ^b	65.95 ± 10.33 ^b
	30	1.43 ± 0.00 ^b	0.11 ± 0.16 ^a	35.89 ± 4.63 ^a	64.75 ± 1.34 ^b
	60	n.d. ^c	2.09 ± 1.16 ^a	45.24 ± 3.97 ^a	103.45 ± 10.02 ^a
	120	n.d. ^c	0.82 ± 0.31 ^a	2.45 ± 0.11 ^c	22.10 ± 0.97 ^c

Table 3 (Part B). Vitamins D and photoisomers detected in the SFE extract during 2 h irradiation using different UV-lamps. Provitamin D₄ (PRO₄), previtamin D₄ (PRE₄), tachysterol₄ (T₄), lumisterol₄ (L₄) and vitamin D₄ (V₄). n.d. = not detected.^{a-c} Different letters denote significant differences ($P < 0.05$) between different times of exposure for the same compound and the same lamp.

Lamp	Time (min)	PRO ₄ (mg/g)	PRE ₄ (mg/g)	T ₄ (mg/g)	L ₄ (mg/g)	V ₄ (mg/g)
UV-C (254 nm)	0	n.d. ^b	n.d. ^a	n.d. ^a	n.d. ^b	n.d. ^a
	15	n.d. ^b	n.d. ^a	n.d. ^a	0.38 ± 0.42 ^b	n.d. ^a
	30	1.13 ± 0.00 ^a	n.d. ^a	n.d. ^a	1.67 ± 0.00 ^a	n.d. ^a
	60	n.d. ^b	n.d. ^a	0.27 ± 0.22 ^a	0.16 ± 0.22 ^b	n.d. ^a
	120	n.d. ^b	0.58 ± 0.77 ^a	2.03 ± 2.07 ^a	0.70 ± 0.36 ^a	0.13 ± 0.19 ^a
UV-A (365 nm)	0	n.d. ^a	n.d. ^a	n.d. ^a	n.d. ^a	n.d. ^a
	15	n.d. ^a	n.d. ^a	n.d. ^a	0.26 ± 0.37 ^a	n.d. ^a
	30	n.d. ^a	n.d. ^a	n.d. ^a	0.55 ± 0.40 ^a	n.d. ^a
	60	n.d. ^a	n.d. ^a	n.d. ^a	0.22 ± 0.31 ^a	n.d. ^a
	120	n.d. ^a	n.d. ^a	n.d. ^a	0.68 ± 0.37 ^a	n.d. ^a
WS-UV	0	n.d. ^a	n.d. ^a	n.d. ^b	n.d. ^b	n.d. ^c
	15	0.03 ± 0.04 ^a	0.63 ± 0.04 ^a	1.01 ± 0.57 ^b	1.32 ± 0.55 ^a	1.05 ± 0.32 ^c
	30	n.d. ^a	0.83 ± 1.18 ^a	1.34 ± 0.08 ^a	1.36 ± 0.02 ^a	3.43 ± 0.16 ^b
	60	n.d. ^a	1.65 ± 0.04 ^a	2.18 ± 0.04 ^a	1.53 ± 0.08 ^a	7.97 ± 0.44 ^a
	120	n.d. ^a	n.d. ^a	n.d. ^b	0.20 ± 0.12 ^b	0.38 ± 0.19 ^c

Since irradiation with WS-UV lamp was promoting generation of other structures (vitamin D₄ -related) and the aim of the work was to increase the level of vitamin D₂ but avoiding the complete ergosterol transformation (because of its hypocholesterolemic properties) the WS-UV lamp was discarded and the UV-C lamp was selected to carry out further experiments.

Effect of the solvent and temperature utilized for SFE-extracts irradiation

Transformation of ergosterol from SFE extract into vitamin D₂ under UV-C light was also studied using different solvents. After 1 h irradiation (at 50 °C), vitamin D₂ was slightly synthesized if water was used as reaction medium (Figure 4). Addition of methanol up to different percentages improved the transformation ratio but up to levels significantly lower than when irradiation was carried out using 100% organic solvents such as methanol or ethanol suggesting that solubilization of the SFE extract was essential for the proper vitamin D₂ generation.

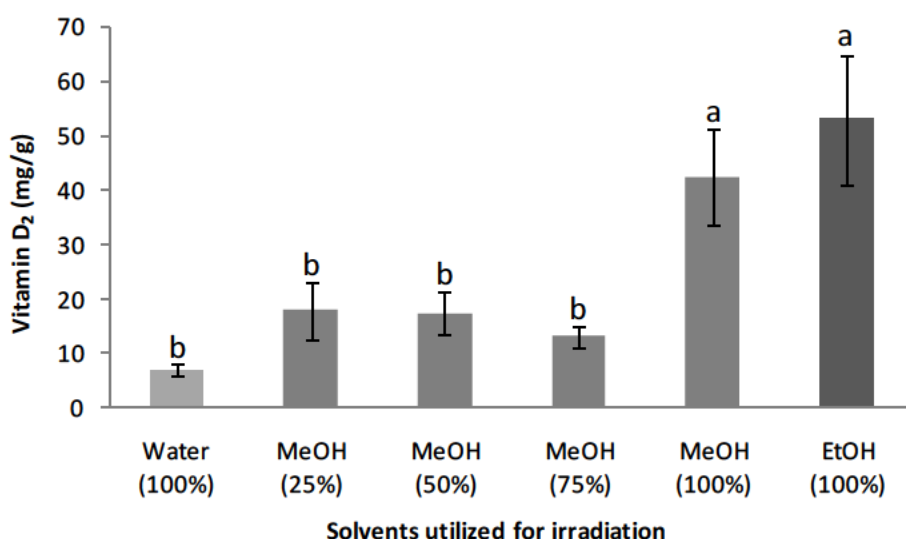


Figure 4. Influence of the different solvents (utilized to dissolve SFE extract for UV irradiation) in vitamin D₂ levels. ^{a-b} Different letters denote significant differences ($P < 0.05$).

Because of the detrimental effect noticed on sterol content and vitamin D₂ transformation after prolonged WS-UV irradiation, the effect of temperature was also studied on SFE extracts irradiated with the UV-C lamp to ensure that the observed degradation could be due to the heat generated by the IR rod and to estimate the optimal temperature needed to enhance the vitamin D₂ generation.

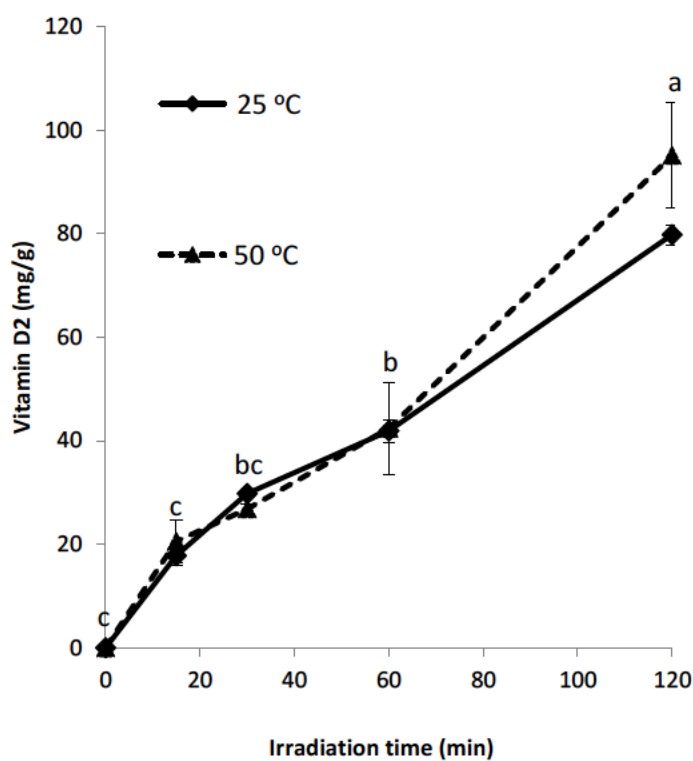


Figure 5. Influence of the different temperatures (utilized during SFE extract UV irradiation) in vitamin D₂ levels. ^{a-c}Different letters denote significant differences ($P < 0.05$) between different irradiation times at the same temperature (25 °C or 50 °C).

However, no significant differences were found after 1 h UV-C irradiation if temperatures were maintained from 25 up to 50 °C (Figure 5). Other tested temperatures (1 h) such as 30, 40 and 60 °C did not significantly improve speed of vitamin D generation (as also noticed by Wittig et al. (2013) after irradiation of oyster mushrooms fruiting bodies) [6]. However, after 2 h irradiation of the SFE extract at 50 °C, a slightly higher amount of vitamin D₂ was obtained than at 25 °C.

Effect of the distance to UV source

The optimal distance to the UV source was also investigated because previous studies placed mushroom fruiting bodies at many different distances (from 10 up to 30 cm) with no further testing to study whether they were the more adequate

position for irradiation. For instance, Huang et al. (2015) irradiated Oyster mushrooms with a UV-B lamp at 25 °C, for 2 h, at 19 cm far from the light and obtained 69 µg/g vitamin D [22] while Wittig et al. (2013) irradiated the same mushrooms with a similar UV-B lamp at 20 and 30 °C at 10 cm from the light and obtained larger amounts (80 µg/g) with only 10 min irradiation [6]. Differences between both experiments could be due to the fact than the latter mushrooms were placed closer to the UV lamp. Thus, SFE extracts were dissolved in methanol and placed at three different positions from the lamp (Figure 6).

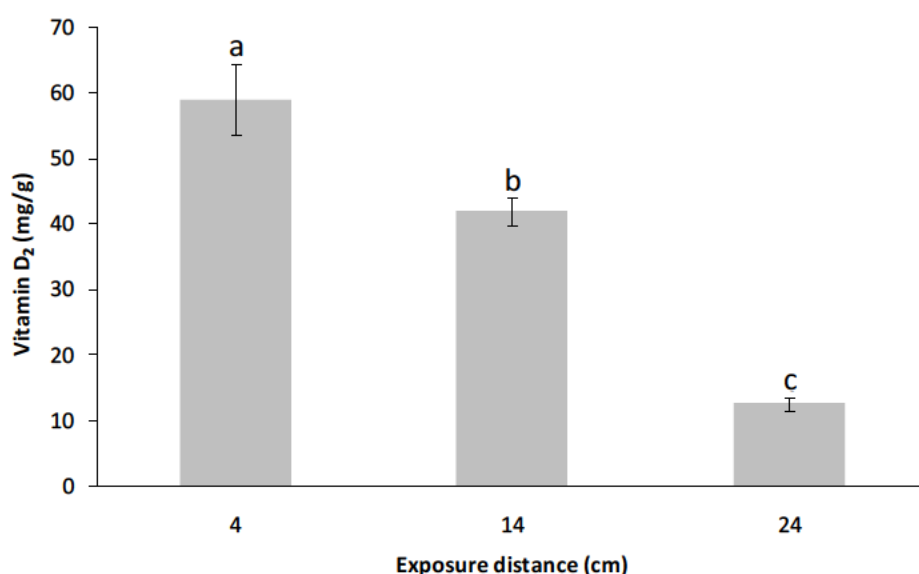


Figure 6. Influence of the different distances from the UV lamp (utilized during SFE extract UV irradiation) in vitamin D₂ levels. ^{a-c} Different letters denote significant differences ($P < 0.05$).

Results indicated that the transformation of ergosterol into vitamin D₂ was enhanced when the vials were placed closer to the UV source since at 4 cm distance almost 5 fold more vitamin D₂ was obtained than at 24 cm after 1 h exposure.

Conclusions

Fractions containing up to 18% (w/w) ergosterol and other ergosterol derivatives can be obtained by supercritical fluid extractions from *Lentinula edodes*.

They can be further processed to induce partial transformation of this provitamin D₂ into vitamin D₂ by UV-light irradiation. Then, the SFE extracts should be dissolved in organic solvents such as methanol or ethanol, exposed at room temperature under WS-UV or UV-C rather than UV-A light and as closer as possible to the UV source. WS-UV irradiation also induced vitamin D₄ formation although in lower amounts than vitamin D₂ or lumisterol₂. However, if WS-UV lamp is couple to IR rod, exposures longer than 1 h are not encouraged due to detrimental effect on ergosterol and vitamin D₂ contents. This procedure to generate vitamin D₂ enriched extracts is > 100 folds more effective than direct fruiting body irradiation followed by extraction.

Acknowledgements

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Manuscript 2

***In vitro* and *in vivo* testing of the hypocholesterolemic activity of ergosterol- and β - D-glucan-enriched extracts obtained from shiitake mushrooms (*Lentinula edodes*)**

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Abstract

Herein, a supercritical extraction plant (with a 6 L extraction cell) was successfully used to obtain ergosterol-enriched extracts from *Lentinula edodes* under the following conditions: a temperature of 40 °C, pressure of 22.5 MPa, reaction time of 1-5h, and the flow rate of 20 L/h for recirculated CO₂). Moreover, ergosterol (ERG) and the SFE extract (SFE) with highest ergosterol concentration were microemulsified and submitted to *in vitro* digestion to study their ability to displace cholesterol from dietary mixed micelles (DMMs). ERG was also mixed with a β -D-glucan-enriched (33.5%) extract (BGE) from *L. edodes* to investigate the synergies between them; the results indicated that all these extracts (including BGE without ERG) could reduce the cholesterol levels in DMMs. However, when ERG and SFE were simultaneously administrated to mice with a hypercholesterolemic diet, no significant differences in the serum cholesterol levels were detected as compared to the case of the control. However, when only BGE was administrated to another mice model previously induced with hypercholesterolemia, significant reduction in the cholesterol levels was noticed.

Introduction

High levels of total cholesterol (TC) and particularly LDL-cholesterol constitute one of the most relevant risk factors of cardiovascular diseases that are still leading the causes of death, particularly in developed countries. However, before treatment with therapeutic drugs, for patients with milder hypercholesterolemia, regular consumption of specific functional foods may be more desirable; nowadays, products including phytosterols or cereal β -D-glucans, exhibit health benefits as hypocholesterolemic products approved by most of the regulatory institutions such as European Food Safety Authority (EFSA), Food and Drugs Administration (FDA), and they are easily found in the supermarkets; however, novel or/and more effective products still need to be developed [1, 2].

Edible mushrooms have been reported as one of the foods with potential hypocholesterolemic activities [3]. They contain fungal sterols that (as noticed for phytosterols) may displace cholesterol from dietary mixed micelles (DMMs) [4]. Dietary cholesterol is mainly absorbed by intestinal enterocytes when incorporated into these small micelles together with the rest of lipid compounds. They are formed during digestion when pancreatic lipases, bile acids, lecithin and other molecules transform the dietary fat into oily drops and large vesicles; the size of these oily drops and large vesicles is reduced until micelles are formed, generating a particular emulsion, and only the micelles with optimal dimensions are incorporated into the cell membranes and thus called DMMs. The larger-sized vesicles are usually excreted together with the precipitated compounds eliminated from the micelles. The lipids provided by the DMMs are only assimilated by enterocytes when specific transporters recognize them and transfer them to the endoplasmic reticulum. Apparently, for cholesterol absorption, the Niemann-Pick C1-like 1 protein is necessary, which shows high affinity for this molecule; thus, non-cholesterol sterols are incorporated in very low amounts as compared to cholesterol (approx. 2 – 5% vs. 60%) [5,6].

The bioavailability of fungal sterols, such as ergosterol, could be enhanced by loading them onto specific microemulsions, improving, for instance, their anti-tumor properties [7]. Microemulsions are thermodynamically stable nanometer systems used

to stabilize compounds difficult to solubilize in physiological fluids. In addition to acting as cholesterol displacers in the DMMs, plant or fungal sterols have been described as modulators of the genes (SREBP, NR1H3 (LXR) FDFT1 (SQS), etc.) involved in the cholesterol metabolism, inhibitors of specific enterocytic enzymes needed for cholesterol absorption and transport (SOAT, MTT, APO48 etc.), liver X receptor (LXR) agonists, etc. [8, 9, 10]. However, at present, it is still unclear whether plant/fungal sterols need to be absorbed in higher concentrations to exert their hypocholesterolemic activities since when they are taken in large amounts, they also induce disorders such as sitosterolemia [11]. Moreover, if they are loaded onto microemulsions, they might have more difficulties to be incorporated in the DMMs and hence to displace cholesterol.

On the other hand, the inclusion of fungal β -D-glucan extracts into ergosterol supplemented food matrices appeared to improve the cholesterol displacement from DMMs [4]. These polymers also show hypocholesterolemic activities although their mechanisms of action appears to be different from those postulated for sterols. These polysaccharides show gel forming properties and they may increase viscosity during digestion, stimulating cholesterol excretion through faeces. They have also been reported as bile acids scavengers, enhancing the transformation of cholesterol to re-establish their levels. Moreover, the fermentation of β -D-glucans by colonic microbiota appears to stimulate the production of short-chain fatty acids that may block cholesterol biosynthesis by inhibiting the HMGCR, a key enzyme in the cholesterol pathway [12].

Ergosterol-enriched extracts can be successfully obtained from natural sources, such as mushrooms e.g. *Lentinula edodes* (Chapter 1, Manuscript 1) [13], *Agaricus bisporus* [14] or *Agaricus brasiliensis* [15], using supercritical CO₂ extraction. The process is more effective than the use of organic solvents or other advanced technologies such as pressurized liquid-extraction or microwave/ultrasound-assisted technologies [14, 16, 17]. However, the SFE plants utilized in most of the previous publications are usually small or pilot plant scale where extraction conditions can be very easily controlled but lower amounts of extract are obtained as compared to the plants using higher volume extraction cells. Hence, to

obtain extracts in large quantities for animal or clinical trials, a large-scale SFE plant should be utilized and some adjustments should be taken into consideration.

In this study, a large-scale SFE plant was used to obtain sufficient amounts of ergosterol-enriched extract from *Lentinula edodes* to carry out *in vitro* and *in vivo* tests to study its hypocholesterolemic activities. The obtained extract was loaded onto a microemulsion and mixed with a fungal β -D-glucan-enriched extract to investigate whether the microemulsion and the fungal extract could enhance or interfere with the activity of the obtained extract as a cholesterol displacer from DMMs during an *in vitro* digestion model; moreover, two different mice models were utilized to evaluate the *in vivo* hypocholesterolemic effect of the SFE extract and the β -D-glucan-enriched extract.

Materials and Methods

Biological material, standards and reagents

Powdered *Lentinula edodes* S. (Berkeley) fruiting bodies (particle size < 0.5 mm, moisture < 5%) were purchased from Glucanfeed S.L. (La Rioja, Spain) and stored in the dark at -20 °C until use. Commercially available lard (Iberian pork lard, 99.7 g /100 g fat) was purchased from a local supermarket and maintained at 4 °C until use. All the experiments were performed from the same lot.

Solvents such as hexane (95%), chloroform (HPLC grade), methanol (HPLC grade) were obtained from LAB-SCAN (Gliwice, Poland) and absolute ethanol was obtained from Panreac (Barcelona, Spain). Calcium chloride, hydrochloric acid (37%), pepsin (from porcine pancreatic mucosa), sodium hydroxide, sodium chloride, Trizma base, maleic acid, pancreatin (from porcine pancreas), L- α -phosphatidyl choline (lecithin), Sepharose® 4B, phenol, ascorbic acid, BHT (2,6-Di-tert-butyl-*p*-cresol), Kolliphor®EL, and cholesterol (96%) and ergosterol (95%) (used as standards) were purchased from Sigma-Aldrich Química (Madrid, Spain). Carbon dioxide (99.99% purity) was supplied by Air-Liquid España, S.A. (Madrid, Spain).

Supercritical fluid extractions (SFE)

Extractions with CO₂ were carried out in a large scale SFE plant (Zean Consultores S.L., Madrid, Spain) comprising a 6 L cylinder extraction vessel and two different separators (S1 and S2) of 1.6 L capacity each with independent control of temperature and pressure. A detailed explanation of the experimental device can be found in the literature [18]. The CO₂ flow rate was set at 20 L/h, and during the total extraction time tested herein (from 1 to 5 h), CO₂ was recirculated. The temperature was fixed at 40 °C in the extraction vessel and the separators, and the extraction pressure was maintained at 22.5 MPa. Ethanol was not used as a co-solvent because previous studies had indicated that although higher yields could be obtained, the use of only CO₂ was more selective in generating fractions with higher sterol concentrations [14]. The fractions extracted in 2 separators (S1 and S2) were obtained with ethanol and immediately submitted to drying using a rotary vacuum evaporator. Ethanol was utilized based on previous studies [18] due to its following advantages: it is an organic solvent and easily evaporates *via* mild processes and it can completely solubilize the separated fractions by detaching the extracts from the separator walls. The dried extracts were stored at -20 °C until further analysis.

Preparation of ergosterol and SFE extract microemulsions

Sterol-loaded microemulsions were formulated as indicated by Yi et al. (2012) [7] with slight modifications. Briefly, ergosterol (50 mg) or a selected SFE extract containing 52.5% ergosterol (SFE) (95 mg) were mixed with lard (1 g). Subsequently, Koliphor EL (21% w/w) and 5% ethanol were mixed thoroughly and stirred as a surfactant-cosurfactant solution followed by mixing with 3% of the supplemented lards. Then, water (71%) was added dropwise to the oily mixtures under gentle stirring to generate microemulsions: MERG (microemulsified ergosterol) and MSFE (microemulsified SFE extract) were prepared and immediately used in the *in vitro* digestion model.

Preparation of a β -D-glucan-enriched (BGE) extract

A β -D-glucan-enriched extract (BGE) was prepared by mixing different polysaccharides fractions, such as a fraction (2.5%) containing water soluble β -D-glucans (named ExA in Chapter 4, Manuscript 1), a fraction (26%) extracted with hot water (98 °C), filtered through a multichannel ceramic membrane (Ceramem Corporation, Waltham, USA) and concentrated with a spiral wound Nanomax50 membrane (Millipore, Bedford, USA) (called RF2 in Chapter 3, Manuscript 3) and the remaining fraction (71.5%) containing mainly chitins and insoluble β -D-glucans (see Chapter 3, Manuscript 3), with hypocholesterolemic activities. The fractions were lyophilized, pooled together and stored at -20°C until further use. The resulting mixture contained 33.5% (w/w dry weight) β -D-glucans and 0.23% ergosterol (determined as described in Chapter 4, Manuscript 1).

***In vitro* digestion and isolation of the dietary mixed micelles (DMMs)**

Lard supplemented with cholesterol (25 mg/g) was used as a food matrix to evaluate the hypocholesterolemic activity of the different extracts in an *in vitro* digestion model, as reported by Gil-Ramirez et al. (2014) [4]. The hypercholesterolemic lard was mixed with ergosterol (ERG) (50 mg/g), MERG (32.3 g/g), SFE (95 mg/g) or MSFE (32.3 g/g) as ergosterol-containing formulations and/or BGE extract (149 mg/g) as a β -D-glucan plus ergosterol-containing extract (the indicated concentrations were adjusted to include 50 mg/g ergosterol and/or β -D-glucans in the food matrix). The mixtures were gently stirred at their melting temperatures until complete incorporation of the supplements into the lipidic matrices occurred.

The supplemented food matrices (1 g) were submitted to *in vitro* digestion following the procedure described by Gil-Ramirez et al. (2014) [4]. After this, the fraction containing the dietary mixed micelles (DMMs) was isolated using a Sepharose®4B column with 0.15M NaCl/16 mM bile salts as the mobile phase, as indicated by their intermicellar bile salt concentration (IMBC). The DMM fractions (16 mL) were identified because their cholesterol fraction (determined by an enzymatic SpinReact cholesterol quantification kit (SpinReact SAU, Girona, Spain)

co-eluted with their phospholipids concentration (determined using an enzymatic Wako kit (Wako, Madrid, Spain)) at the proper elution volume [4].

Sterols quantification by GC-MS-FID

Sterols were extracted from the samples and quantified following the procedure described by Gil-Ramirez et al. (2013) [14]. The unsaponified fractions were injected into the Agilent 19091S-433 capillary column (30 m x 0.25 mm ID and 0.25 μ m phase thickness). The column was connected to a 7890A System gas chromatograph (Agilent Technologies, Santa Clara, USA) including a G4513A auto injector and a 5975C triple-axis mass spectrometer detector. The injector and detector conditions as well as the column temperature program were same as those described by Gil-Ramirez et al. (2013) [14]. Cholesterol and ergosterol were used as standards, and hexadecane (10% v/v) was used as an internal standard.

The GC-MS database identified the obtained peaks based on previous studies ([14, 19, 20]. The major detected sterols were cholesterol (RT = 11.9 min), ergosterol (ergosta-5,7,22-trien-3 β -ol) (RT= 12.6 min), ergosta-7,22-dienol (RT =12.8 min), ergosta-5,7-dienol (RT = 13.1 min) and ergosta-7-enol (fungisterol) (RT= 13.3 min).

Animal trials

The male mice C57/BL6JRj (6 weeks old; Janvier SAS, Le Genest-Saint-Isle, France) were maintained under the following in temperature-, humidity- and light-controlled conditions: 24 \pm 2 $^{\circ}$ C, 40-60% humidity, 12:12 hour light:dark cycle, respectively; moreover, they had free access to water and food. In addition, two different experiments were carried out, and both were approved by the Institutional Animal Welfare and Ethics Committee of La Paz University Hospital (Madrid, Spain) according to the current Spanish and European legislation (RD53/2013 and EU 63/2010).

In the first experiment, 35 mice were randomly divided into four groups. The group NC (normal control, n = 5) was fed a control diet (standard diet, Safe Rodent Diet, A04, Augy, France), the group HC1 (high cholesterol, n = 10) was administrated a high fat hypercholesterolemic diet (HFHD) (standard diet supplemented with 1.25%

cholesterol, 0.5% cholic acid and 12% lard), the group ERG ($n = 10$) was administered the HFHD supplemented with ergosterol (0.45%) and the group SFE ($n = 10$) was fed HFHD supplemented with the SFE extract (up to 0.45% ergosterol in the diet). Blood samples were obtained by mandibular puncture at the beginning and the end of the study (5 weeks), and plasma samples were stored at $-20\text{ }^{\circ}\text{C}$ until further use.

In the second experiment, 20 mice were fed the standard diet supplemented with 1.25% cholesterol and 0.5% cholic acid, and the blood samples were obtained at the beginning and 3 weeks after the feeding started. After 4 weeks, animals were randomly divided into two groups ($n = 10$): one group was administered the same diet and used as control (HC2) and the other (BGE) was administered the diet supplemented with 6% BGE. Both were fed the diet during 5 weeks.

After the feeding periods, mice were obtained from both experimental groups and killed by intracardiac exsanguination under anesthesia with 1.5 % isoflurane; moreover, plasma was obtained and stored at $-20\text{ }^{\circ}\text{C}$ before use. Livers, fat and kidneys were removed, immediately frozen in liquid nitrogen and stored at $-80\text{ }^{\circ}\text{C}$. The levels of total cholesterol, triglycerides, HDL-cholesterol and LDL-cholesterol in the plasma were measured using the Covas C311 Autoanalyzer (Roche, Spain).

Statistical analysis

Differences were evaluated at 95% confidence level ($P \leq 0.05$) using one-way analysis of variance (ANOVA) followed by the Tukey's Multiple Comparison test. Statistical analysis was performed using the SPSS V.13.0 software (SPSS Institute Inc., Cary, NC) and GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA, USA).

Results and discussion

Upscaling of SFE extractions

Large-scale supercritical CO_2 extractions were carried out to scale up the production of the SFE extracts from shiitake mushrooms since similar extracts

obtained from *Agaricus bisporus* decreased the TC/HDL ratio in hypercholesterolemic mice when applied at low concentrations. In this regard, Gil-Ramirez et al. (2016) [9] concluded that to improve their hypocholesterolemic activity, the SFE extracts should be utilized at higher concentrations for *in vivo* testing.

The extraction yields increased with an increase in the extraction time up to 3h, reaching the value of 0.53% taking into consideration the material obtained in both separators (S1+S2) (Figure 1). However, when longer extraction times were used (up to 5h), only a slight increase in the extraction yield was noticed (0.56 % yield) the additional 2 h extraction period. The S1 separator collected more material than the S2 separator (2.3-2.8 folds) regardless of the extraction time. Previous studies reported on the extractions from shiitake mushrooms using a pilot scale plant and similar conditions (22.5 MPa, 35 °C, 3 h) showed higher yields (1.25%) (Chapter 1, Manuscript 1). These yields could be improved in pilot-plants by increasing temperature (2.3% at 22.5 MPa and 75°C) or pressure (2.1% at 35 MPa and 40 °C). However, other researchers obtained similar values in the shiitake SFE at 40 °C during 3 h of extraction under 20 and 25 MPa (0.65 and 0.81%, respectively) using a smaller plant with a 100 mL extraction cell [13].

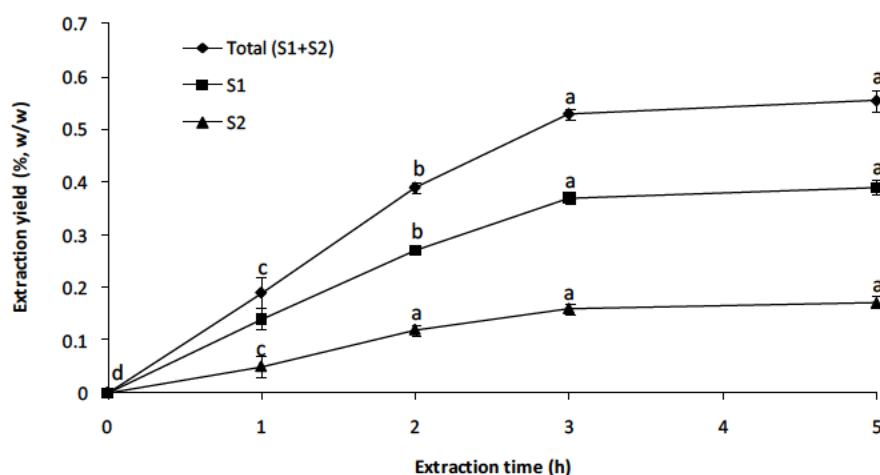


Figure 1. SFE yields obtained in separator 1 (S1), separator 2 (S2) and total (S1+S2) at different extraction times.

The obtained SFE extracts contained up to 53% ergosterol (Table 1), similar to the value indicated by Gil-Ramirez et al. (2013) [14] for *A. bisporus* but significantly higher than others noticed for *L. edodes* using a smaller capacity pilot plant (Chapter 1, Manuscript 1). The results showed that although the yield in this large-scale plant was lower, the extraction was apparently more selective towards sterols than the case when a pilot plant was used. Moreover, when the ergosterol content was determined in the extracts collected in both separators, the S1 extracts exhibited higher cholesterol concentrations (41-53%) than the S2 extracts (19-26%) and statistically significant differences were found in S1 between 1 and 5 h of extraction time suggesting that time could influence ergosterol enrichment of the extracts.

Ergosterol derivatives were also detected in the extracts, although at lower concentrations (Table 1). Fungisterol concentrations were higher than ergosta-5,7-dienol and ergosta-7,22-dienol, and it was distributed within the separators in a similar ratio to ergosterol. However, ergosta-7,22-dienol was specifically recovered in S2, and the concentration of ergosta-5,7-dienol showed no differences between separators. Thus, the extract containing a larger amount of fungal sterols (58.7%) obtained after 5h in S1 was selected as SFE extract to carry out further experiments.

Table 1 (Part A). Fungal sterols content in SFE extracts recovered in separator 1 (S1) and separator 2 (S2) at different extraction times (1, 2, 3 and 5 h). n.d. = not detected; ^{a-c}Different letters denote significant differences ($P < 0.05$) between different extraction times for the same sterol.

Extraction time (h)	Ergosterol (%)		Ergosta-7,22-dienol (%)	
	S1	S2	S1	S2
1	41.44±3.40 ^b	21.52±2.31 ^c	n.d. ^b	1.19±0.28 ^a
2	46.30±1.49 ^{ab}	20.30±0.33 ^c	n.d. ^b	1.04±0.23 ^a
3	46.12±2.06 ^{ab}	18.56±1.03 ^c	n.d. ^b	1.08±0.19 ^a
5	52.50±1.76 ^a	25.90±0.18 ^c	n.d. ^b	1.54±0.07 ^a

Table 1 (Part B). Fungal sterols content in SFE extracts recovered in separator 1 (S1) and separator 2 (S2) at different extraction times (1, 2, 3 and 5 h). ^{a-c}Different letters denote significant differences ($P < 0.05$) between different extraction times for the same sterol.

Extraction time (h)	Ergosta-5,7-dienol (%)		Fungisterol (%)	
	S1	S2	S1	S2
1	0.61±0.13 ^{ab}	0.22±0.05 ^b	4.15±0.19 ^b	2.31±0.23 ^c
2	0.48±0.02 ^{ab}	0.16±0.03 ^b	3.83±0.05 ^{bc}	1.83±0.45 ^c
3	0.46±0.07 ^{ab}	0.40±0.11 ^b	3.92±0.13 ^{bc}	2.76±0.49 ^c
5	0.71±0.09 ^a	0.65±0.02 ^{ab}	5.50±0.36 ^a	3.79±0.09 ^{bc}

***In vitro* testing of the hypocholesterolemic properties of obtained extracts**

Previous *in vitro* studies indicated that ergosterol and, in particular, an SFE extract containing fungal sterols from *A. bisporus* were as effective as β -sitosterol in the displacement of cholesterol from DMMs when they were incorporated in a food matrix such as lard [4]. To test the ability of the SFE extract obtained from *L. edodes*, the same *in vitro* digestion model was reproduced. The results indicated that the DMM fraction generated after the digestion of the lard supplemented with cholesterol and ergosterol (ERG) contained approx. 63% less cholesterol than the case when only cholesterol was added (Figure 2). This reduction was slightly higher than the 49% noticed by Gil-Ramirez et al. (2014) [4]; however, when the SFE extracts were added, this effect was almost the same (69% and 67%); this indicated that regardless of the mushroom utilized, the SFE extracts were able to reduce the cholesterol levels in the DMM fraction (the fraction usually absorbed by intestinal enterocytes).

Moreover, according to previous studies, the bioavailability of phytosterols as well as other non-cholesterol sterols is very limited [8]; however, other studies have indicated that ergosterol bioavailability may be enhanced by loading the extracts onto microemulsions [7]; on the other hand, if sterols are

incorporated into artificial vesicles followed by their incorporation into food matrices, they may be more bioavailable but perhaps would not be able to act as cholesterol displacers in the DMMs. Therefore, ergosterol and the SFE extract were emulsified (MERG and MSFE, respectively), supplemented to the hypercholesterolemic lard and digested to investigate the content of the generated DMM fractions. The results indicated that both extracts could displace cholesterol from the generated DMMs as their corresponding non-microemulsified extracts.

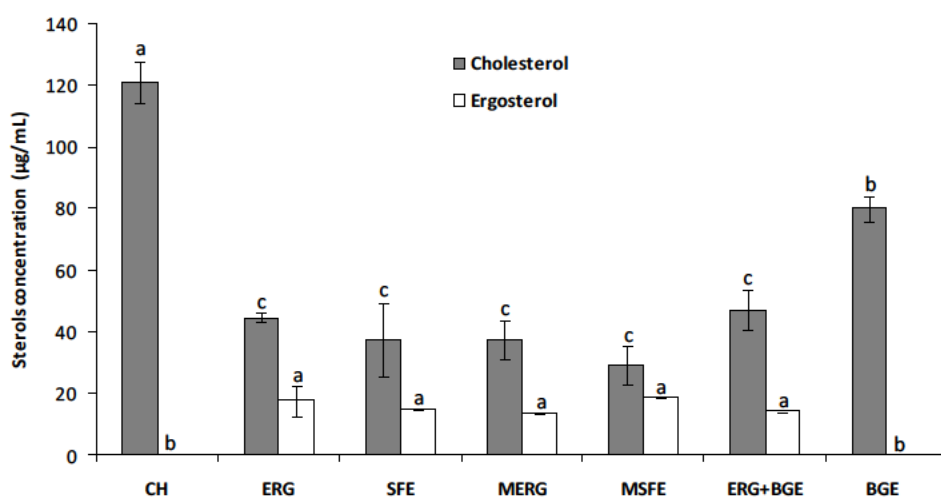


Figure 2. Cholesterol and ergosterol concentrations in the isolated DMM fractions generated after *in vitro* digestion of lard supplemented with cholesterol (CH) and cholesterol together with ergosterol (ERG), SFE extract (SFE), microemulsified ergosterol (MERG), microemulsified SFE extract (MSFE), ergosterol+ β -D-glucan-enriched extract (ERG+BGE) and only β -D-glucan-enriched extract (BGE).

^{a-c} Different letters denote significant differences ($P < 0.05$) between different samples for the same compound.

Other studies suggested that the presence of β -D-glucans extracts obtained from *Pleurotus ostreatus* in the food matrix functionalized with ergosterol reduced approx. 52% more cholesterol content of the generated DMMs than the case when only the sterol was added [4]. When similar food mixture was prepared but using a β -D-glucan-enriched extract obtained from *L. edodes* (BGE+ERG), a lower cholesterol

level was noticed in the generated DMM fraction than that in the DMM control (CH), however, no significantly different values were noted as compared to the case of the DMM fraction generated when the food matrix was supplemented only with ERG. Differences with previous studies might be due to the structural differences between the polysaccharides obtained from both mushroom species or because of their specific extract compositions. Surprisingly, when the BGE extract was also tested without any sterol addition, a significant cholesterol reduction was also noticed (33.7%) in its DMM fraction as compared to that in the control. Since the ergosterol levels in the BGE extract were really low (0.23%), the noticed hypocholesterolemic activity might be because of its high β -D-glucans content (33.5%). These polymers were able to scavenge other small molecules such as bile acids or cholesterol in their structures [21, 22]. In fact, it is suggested as their potential mechanism for lowering the cholesterol levels in serum [12] although other mechanisms may be involved [23].

Ergosterol was also detected in the generated DMM fractions, but it was always incorporated in lower concentrations than cholesterol (on average 58% less); this was in agreement with previous results [4]; moreover, no significant differences were noticed between the different tested extracts; this suggested that the incorporation of ergosterol in the DMM had a significant effect on the ergosterol content in the food matrix than on the presence of other components (such as β -D-glucans and surfactants) in the mixture because all the extracts were supplemented to the hypercholesterolemic lard at different concentrations to ensure the achievement of the same ergosterol content.

***In vitro* testing of the hypocholesterolemic properties of obtained extracts**

Animal studies were carried out to confirm the hypocholesterolemic activity noticed *in vitro* for the extracts. However, to minimize the number of mice utilized in this experiment, only ergosterol as a standard compound and the SFE extract were tested since no significant differences were found with the MERG or MSFE in terms of their capacity to displace cholesterol from DMMs. The MSFE preparation included more processing that might increase the cost if up scaled. Moreover, the administration of the preparations and the induction of hypercholesterolemia were

simultaneously carried out to study the potential of these preparations as preventive more than palliative formulations since the latter influence was already investigated for a SFE extract obtained from *A. bisporus* [9].

The mice groups treated with normal diet (NC) or hypercholesterolemic diet (HC1) used as control as well as the ERG and SFE groups showed similar values of total cholesterol (TC) (91.4 ± 10.8 mg/dL on an average), HDL (1.9 ± 0.7 mmol/L), LDL (0.2 ± 0.1 mmol/L), triglycerides (TG) (158 ± 68.8 mg/dL), AST (74.8 ± 36.1 UI/dL), ALT (18.0 ± 8.1 UI/dL) and glucose 50.0 ± 27.5 (mg/dL) at the beginning of the experimental period. However, after 5 weeks, only the NC group maintained these levels, and the rest of groups exhibited increased TC levels with non-significant differences between HC1 (230.3 ± 67.1 mg/dL), ERG (277.3 ± 35.5 mg/dL) or SFE (262 ± 52.6 mg/dL) groups. The increase noticed in ERG group was more influenced by a high LDL value (5.7 ± 1.0 , whereas HC1 showed 4.6 ± 1.7 mmol/L) and that noticed in the SFE group was more influenced by a high HDL value (3.2 ± 1.0 , whereas HC1 was 2.2 ± 0.3 mmol/L); however, differences in both parameters were insignificant.

Table 2 (Part A). Total (TC), HDL (HDL) and LDL-cholesterol (LDL) values of mice fed 9 weeks HFHD (HC2) and HFHD supplemented with BGE from week 4 to 9. Blood samples were obtained after 0, 3 and 9 weeks of administration. Asterisks indicate statistically significant differences between control and BGE group (* $P < 0.05$; ** $P < 0.001$).

Time (weeks)	Group	TC (mg/dL)	HDL (mmol/L)	LDL (mmol/L)
0	HC2	43.05 ± 8.96	1.01 ± 0.17	0.15 ± 0.03
	BGE	34.53 ± 14.35	$0.81 \pm 0.24^*$	1.51 ± 2.89
3	HC2	96.68 ± 32.72	1.18 ± 0.22	2.06 ± 0.77
	BGE	97.11 ± 33.03	1.17 ± 0.22	2.22 ± 0.84
9	HC2	259.58 ± 48.26	2.01 ± 0.13	5.60 ± 1.45
	BGE	$162.51 \pm 33.78^{**}$	$1.62 \pm 0.31^*$	$3.62 \pm 0.52^*$

Table 3 (Part B). Triglycerides (TG), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and glucose (GLC) values of mice fed 9 weeks HFHD (HC2) and HFHD supplemented with BGE from week 4 to 9. Blood samples were obtained after 0, 3 and 9 weeks of administration. No statistically significant differences between control and BGE group were noticed.

Time (weeks)	Group	TG (mg/dL)	AST (UI/dL)	ALT (UI/dL)	GLC (mg/dL)
0	HC2	69.26±19.67	44.49±17.19	10.01±2.47	29.78±12.12
	BGE	54.83±23.95	47.39±28.26	8.53±4.71	20.43±9.50
3	HC2	30.03±8.12	113.89±57.61	122.56±120.53	56.08±13.98
	BGE	24.78±9.92	71.26±47.39	95.71±130.31	66.78±17.65
9	HC2	34.68±5.66	119.41±61.69	92.61±60.94	64.69±18.57
	BGE	29.97±3.98	154.61±62.64	146.53±74.06	74.70±27.24

Similarly, the TC and glucose values increased in all the groups (from 40.5 to 45.6 mg/dL and from 155.5 to 192.7 mg/dL, respectively). Therefore, the ergosterol-containing preparations showed no hypocholesterolemic activity *in vivo*. These results differ from those reported in the recent studies carried out in rats fed simultaneously a hypercholesterolemic diet supplemented with 0.5% or 1.5% ergosterol for 8 weeks [24] or previous tests carried out in mice but where first hypercholesterolemia was induced followed by treatment with ergosterol-containing preparations obtained from *A. bisporus* [9].

Thus, since the BGE extracts also showed hypocholesterolemic activity *in vitro* but their mechanism of action might be different from that of fungal sterols, another *in vivo* experiment was carried out to investigate whether the β -D-glucan-enriched extract was more effective than the fungal sterols. This time, the experimental setting was changed; moreover, at first, the mice were fed a hypercholesterolemic diet, and then, they were administrated the BGE extract together with unhealthy diet to investigate the effect of the extracts as palliative compounds. The results indicated that the administration of the standard diet supplemented with cholesterol and cholic acid for 3 weeks increased TC, HDL and

LDL levels in all the mice, inducing hypercholesterolemia (Table 2). The glucose concentration and hepatic enzymes activities were also influenced, and their values were increased. Only the TG amounts remained unchanged. After 4 weeks, the HC2 group continued with the hypercholesterolemic diet, and the BGE group diet was supplemented with the BGE extract for 5 weeks. After this feeding period, the BGE group exhibited significantly lowered their TC values, more than 1.5 fold lower than the HC2 group levels. The reduction might be due to the lower HDL and LDL levels noticed in the BGE group. The different diet did not modify the other determined parameters since the TG, AST, ALT and glucose values were similar in both groups. These results were in line with previous findings where mice fed with lard supplemented with a β -D-glucan extract obtained from *P. ostreatus* prevented the TC increase induced by a hypercholesterolemic diet administrated during 5 weeks [25]. Similar hypocholesterolemic activities were noticed for *A. bisporus*, *Grifola frondosa*, *Flammulina velutipes* and *L. edodes* dietary fibers but on normocholesterolemic rats [26, 27]. However, in other studies where a β -D-glucan-enriched extract (from *P. ostreatus*) was administrated, the BGE (as palliative compounds) did not significantly reduced TC levels in mice serum [28]. The different mushroom species or the administration of β -D-glucans at higher concentrations than those utilized in the study might be the reason for the higher effectiveness of the BGE extract obtained from *L. edodes*.

Conclusions

Herein, ergosterol-enriched extracts were successfully obtained from *Lentinula edodes* fruiting bodies by supercritical CO₂ extraction with levels up to almost 53% ergosterol (approx. 59% of total sterols). The extraction yields almost linearly increased in the extraction time up to 3h; however, the extracts obtained after 5 h contained higher sterol concentrations. The extracts collected in the separators showed slightly different compositions: the S1 extracts had higher ergosterol and fungisterol contents than the S2 extracts. However, ergosta-7,22-dienol was exclusively found in S2. The SFE extract collected after 5 h in S1 and commercial ergosterol were also microemulsified and tested as cholesterol displacers using an *in*

vitro digestion model where the DMM fraction was isolated. Ergosterol and the SFE extract with or without microemulsion succeeded to reduce the cholesterol levels within the generated DMM, showing potential as hypocholesterolemic compounds. However, when they were administrated concomitant with a hypercholesterolemic diet to a mice model, no reduction of serum cholesterol levels was noticed as compared to the control. Perhaps the hypocholesterolemic effect reported for other SFE extracts was only effective when the extracts were administrated as palliative ingredients. These results indicate that although *in vitro* testing may be encouraged to screen biological activities of many bioactive compounds, *in vivo* studies should also be performed as the results may disagree with the *in vitro* results. For instance, the BGE extract obtained from *L. edodes* with low ergosterol content (0.23%) but high β -D-glucan concentration (33.5%) showed lower ability to displace cholesterol in the *in vitro* model but it was able to successfully reduce the cholesterol levels in hypercholesterolemic mice model. Therefore, further investigation is encouraged to test BGE hypocholesterolemic activity. Thus, clinical trials must be carried out before its utilization in novel functional food formulations.

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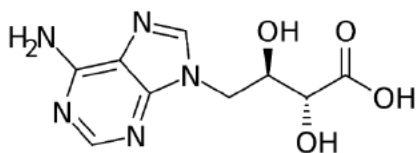
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Chapter 2

Production of eritadenine-enriched extracts



Preface

Eritadenine was another fungal bioactive compound showing antihypertensive properties according to *in vitro* tests and hypocholesterolemic activities in animal trials. Although it was firstly isolated from *L. edodes*, a few reports indicated that it was also present in other mushrooms. Therefore, in order to select the best source to obtain eritadenine-enriched extracts, a screening of 12 different mushroom species was carried out in the work entitled *Effect of traditional and modern culinary processing, bioaccessibility, biosafety and bioavailability of eritadenine, a hypocholesterolemic compound from edible mushrooms*. The results indicated that shiitake was the best source and therefore, the levels of this metabolite were quantified in this species during the different developmental stages and within the different mushroom tissues to define with higher precision the optimal raw material to be submitted to extractions. Two previously described extraction methods were compared and optimized for precise eritadenine quantification.

Shiitake mushrooms or food functionalized with mushroom extracts are not usually consumed raw but submitted to culinary processing that might affect their components including eritadenine. Thus, the effect of traditional cooking (grilling, boiling, frying and microwaving) was evaluated on eritadenine levels. In addition, a few procedures included in the so called *nouvelle cuisine* or *molecular gastronomy* (gelling, texturizing, etc.) that are usually prepared to give to the novel functionalized food a more tempting appearance, were reproduced too since they might also positive- or negatively influence eritadenine stability and bioaccessibility. Afterwards, the culinary preparations were digested in an *in vitro* digestion model to evaluate their bioaccessibility. Moreover, a prepared eritadenine-enriched extract was administrated to rats to evaluate whether it was safe for consumption, if the compound manage to reach its target organ (liver) being then bioavailable and also to confirm its ability as hypocholesterolemic compound.

Manuscript 1

Effect of traditional and modern culinary processing, bioaccessibility, biosafety and bioavailability of eritadenine, a hypocholesterolemic compound from edible mushrooms

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Abstract

Eritadenine is a hypocholesterolemic compound that is found in several mushroom species such as *Lentinula edodes*, *Marasmius oreades* and *Amanita caesarea* (1.4, 0.7 and 0.6 mg per g dry weight, respectively). It was synthesized during all developmental stages, being present in higher concentrations in the skin of shiitake fruiting bodies. When subjected to traditional cooking, grilling followed by frying were more adequate methodologies than boiling or microwaving to maintain its levels. Modern culinary processes such as texturization (with agar-agar) and spherification (with alginate) also interfered with its release. An animal model (where male and female rats were administered 21 and 10 mg per kg animal day of eritadenine) indicated that intake of the compound was safe under these concentrations; it reached the liver and reduced the atherogenic index (TC/HDL) in rat sera. Thus, it might be used to design a functional food.

Introduction

Edible mushrooms are consumed worldwide due to their particular flavors and nutritional values. Their consumption is also encouraged because of their health promoting properties e.g. their ability to lower cholesterol levels in serum. Therefore, certain mushroom species could be used as starting material to design functional foods. They contain fungal sterols and β -D-glucans that can impair dietary cholesterol absorption [1] and other molecules that affect the biosynthesis of endogenous cholesterol [2,3]. They can also be used to synthesize other compounds such as eritadenine that can also indirectly influence serum LDL levels [4].

Eritadenine ((2(*R*),3(*R*)-dihydroxy-4-(9-adenyl)butanoic acid), also named lentysine or lentinacin, is an adenosine analog derived from the secondary metabolism that was firstly isolated from shiitake mushrooms (*Lentinula edodes*) [5]. Several studies demonstrated that its hypocholesterolemic activity in mice and rats was related to its ability as an *S*-adenosyl-L-homocysteine hydrolase (SAHH) inhibitor. Eritadenine modulated hepatic phospholipid metabolism by decreasing the phosphatidylcholine/phosphatidylethanolamine ratio. However, its precise mechanism of action is not yet fully elucidated [4, 6, 7] and therefore certain safety risks might arise if food products are functionalized by adding eritadenine-enriched extracts as hypocholesterolemic ingredients.

Another aspect to take into consideration is the fact that edible mushrooms (or their functionalized foods) are not usually consumed raw but they are subjected to culinary treatments before ingestion. These processes, usually involving heat (boiling, grilling, etc.), might modulate the concentrations of the fungal bioactive molecules that are assimilated [8,9]. Moreover, novel culinary procedures that are nowadays in trend, presenting surprising dishes or food products (gelling, spherification, etc.) to potentiate consumer interest, might influence their levels too since they include food additives in their formulations (hydrocolloids, thickeners, etc.). These additives could directly interact with the bioactive ingredients modifying their bioaccessibility. Later on, the bioactive compounds that survive the culinary treatments might be further modified during mastication, stomach and/or intestinal digestions influencing their absorption levels at the human intestine [10]. However, not many studies have been

carried out to evaluate the influence of all these processes on bioactive compounds such as eritadenine.

Therefore, a screening through several edible mushrooms was carried out to find an interesting eritadenine source. Different fruiting body tissues and developmental stages were also investigated. Then, shiitake mushrooms were subjected to traditional and modern culinary processing to evaluate their effect on eritadenine stability. Afterwards, the effect of digestion on eritadenine bioaccessibility was studied using an *in vitro* digestion model and its toxicity, bioavailability and hypocholesterolemic effect were evaluated with *in vivo* animal experiments.

Materials and methods

Biological material

Mushroom species such as *Lentinula edodes* S. (Berkeley), *Lactarius deliciosus* (Fr.), *Boletus edulis* (Bull. Ex Fr.), *Pleurotus ostreatus* (Jacq.Ex Fr.) Kummer, *Agaricus bisporus* L. (Imbach), *Amanita caesarea* (Scop. Ex Fri.) Pers. Ex Schw., *Cantharellus tubaeformis* (Schaeff) Quel, *Ganoderma lucidum* (Curtis) P.Karst., *Lyophyllum shimeji* (Kawam.), *Morchella conica* (Pers.), *Auricularia auricula judae* (Bull. Ex St.Amans) Berck, *Marasmius oreades* (Bolt. Ex Fr.) Fr were purchased in season from the local market in Madrid (Spain). They were lyophilized and ground into fine powder as described by Ramirez-Anguiano *et al.* (2007) [11]. Dried mushroom powders were stored at -20 °C until further use.

Fresh *Lentinula edodes* fruiting bodies were also subjected to traditional cooking treatments (see later) or processed to separate the different tissues with a knife. Then, individual parts were treated as described above.

In order to test an eritadenine-enriched preparation as potential functional ingredient for the *in vivo* tests, an extract was obtained by stirring *L. edodes* powder with water (0.5 g/L) for 1 min at room temperature. The resulting suspension was centrifuged for 7 min at 7000 rpm at 10 °C and the obtained supernatant was immediately frozen and lyophilized. This fraction was an eritadenine-enriched extract containing 3.1 mg/g.

Reagents

Solvents such as methanol (HPLC grade) and acetonitrile (HPLC grade) were acquired from Lab-Scan (Gliwice, Poland). Ethanol (HPLC grade), diethyl ether (HPLC grade) and calcium chloride were purchased from Panreac (Barcelona, Spain). Hydrochloric acid (37%), trifluoroacetic acid (99%), sodium hydroxide, pepsin (from porcine gastric mucosa), sodium chloride, Trizma base, maleic acid, pancreatin (from porcine pancreas) and L- α -phosphatidyl choline (lecithin) were obtained from Sigma-Aldrich (Madrid, Spain) and D-eritadenine (90%) from SYNCHEM UG & Co. KG (Felsberg, Germany).

All additives used for culinary preparations were food grade: gelatin was acquired from Mondelez International (Madrid, Spain) and agar-agar (E406), sodium alginate (E401) and calcium lactate (E327) from Cuisine Innovation (Dijon, France).

Traditional culinary processing

Lentinula edodes fresh fruiting bodies were cut into slices and cooked (30 g) following four traditional methods: grilling, microwave cooking, frying and boiling. These treatments were carried out as described by Soler-Rivas *et al.* (2009) [9] in quadruplicate and the resulting cooked mushrooms were directly subjected to an *in vitro* digestion model or freeze-dried, ground and stored at -20°C until further analysis.

Modern culinary processing

Lyophilized shiitake fruiting bodies were subjected to modern culinary processes used on molecular gastronomy such as thickening (usually called ‘texturization’), gelling and ‘spherification’.

Powdered shiitake mushrooms (500 mg) were mixed with 50 mL water and 1 g agar-agar (as a thickener) and homogenized with a culinary blender (Minipimer 3MR320 Braun, Aschaffenburg, Germany). Afterwards, the mixture was heated up to 100 °C and maintained for 1 min. Then, an aliquot was collected with a 60 mL syringe and injected into a silicone tube (77 cm length and 0.5 cm diameter). The filled tube was partially folded, making loops, and submerged in an ice bath to cool

down. The semi-solidified (texturized) gel obtained was extracted from the silicone tube by forcing air inside with another syringe. Then, the texturized shiitake powder resembled semi-transparent spaghetti with a mushroom taste.

Powder from the same shiitake batch (500 mg) was added to a previously hydrated gelatin solution obtained by dissolving a gelatin film (1 g) in cold water (50 mL) for 3 min. Then, the mixture was stirred at 75 °C for 2 min. Afterwards, the homogenate was poured into a round mold and stored at 4 °C for 2 h allowing gel formation.

‘Spherification’ was carried out by mixing the shiitake powder (500 mg) and sodium alginate (400 mg) with 50 mL water and homogenizing them with a blender until a slightly viscose solution was obtained. The mixture was kept 5 min at room temperature to eliminate bubbles. Then, it was introduced into a 60 mL syringe and slowly dropped on a calcium lactate solution (44 mM). The surface of the liquid drops polymerized when placed in contact with Ca^{2+} , yielding jelly spheres. Spheres were left for approx. 3 min, transferred to a water bath to remove calcium excess, taken out with a perforated spoon and plated.

Each culinary process was carried out 4 times. The resulting preparations were directly subjected to an *in vitro* digestion model or freeze-dried, ground and stored at -20 °C until further analysis.

***In vitro* digestion model**

Fresh shiitake fruiting bodies, as well as the food preparations resulting from the traditional and modern culinary processes, were digested following the procedure described by Gil-Ramirez et al. (2014) [10] with modifications. Samples (15 g of traditional culinary processed shiitake or the modern preparations including 500 mg shiitake powder) were masticated by a volunteer for approx. 2 min and spat into a beaker. Milli-Q water was acidified with 6 M HCl (adjusting the pH to 2.0) and added (54 mL) to the masticated samples. The mixture was transferred to a thermostatic vessel at 37 °C with mild stirring and pepsin (275 mg) was also incorporated. Then, it was incubated for 1 h and stirred in a titrator device (Titrino plus, Metrohm, Herisau, Switzerland) simulating gastric digestion. Afterwards, intestinal digestion was

initiated by adding 5 mM CaCl_2 and 150 mM NaCl and adjusting the pH to 6.0 by adding 0.5 M NaOH. Then, a pancreatic solution (6 mL) containing 20 mg pancreatin, 633 mg bile extract and 228 mg lecithin (in 50 mM Trizma-maleate buffer pH 7.5) was added and the pH was adjusted to 7.5 and maintained for 2 h using a viscotrode (Metrohm, Herisau, Switzerland) placed in the titrator device. The stirring level and temperature were the same than used in the gastric digestion simulation.

After the digestion process, digested samples were heated in a water bath at 80 °C for 10 min to inactivate digestive enzymes (eritadenine was resistant to this thermal treatment as indicated in Chapter 3, Manuscript 3; Chapter 4, Manuscript 1) and then subjected to centrifugation (7000 rpm 15 min) to separate the supernatant that was considered as the bioaccessible fraction. Eritadenine bioaccessibility was estimated to be the ratio (%) between the amount of eritadenine in the bioaccessible fraction and that in the sample before the digestion process.

Animals and diets

Sprague Dawley adult (5 weeks old) male and female rats ($n = 24$) were purchased from Charles River (San Cugat del Valles, Barcelona, Spain) and housed, separated by sex, in groups of four animals per cage. Animals were maintained under controlled conditions of temperature, humidity and light (24 ± 2 °C, 40–60% humidity, 12 h:12 h light/ dark cycle) and had free access to water and food (commercial rodent maintenance diet A04; Scientific Animals Food & Engineering, Augy, France). After an adaptation period (6 days) animals were weighed and randomly divided into three groups per sex: control group (C) that remained with the standard diet, another group that was fed with a low eritadenine dose (10 mg per kg animal per day) (LE) and the third group was fed a higher dose (HE) (21 mg per kg animal per day). Diets were prepared mixing A04 chow with the corresponding amount of the eritadenine-enriched extract necessary to obtain the indicated eritadenine concentration per group. The nutrient composition of each diet is summarized in Table 1. Animals were maintained on this diet for 5 weeks with daily evaluation of behavioral (posture and activity) and physiological (fur and mucosa status, hydration and the presence of secretions and wounds) parameters were scaled

by trained staff and weekly monitoring of weight gain. Feces were collected at the beginning and at the end of the experimental period and maintained at -20°C until further use.

Table 1. Nutritional composition of diets. Rodent standard diet (A04) supplemented with high and low doses of the eritadenine-enriched extract containing 3.1 mg/g eritadenine. Results are expressed as g/100 g in dry base.

	High Dose	Low Dose	Control
Carbohydrates	54	57	60
Protein	14.4	15.3	16
Lipids	2.7	2.9	3
Dietary fibre	3.6	3.8	4
Eritadenine-enriched extract	10	4.8	----

The protocol was approved by the Institutional Animal Ethics Committee of La Paz University Hospital (Madrid, Spain) and procedures were performed in accordance with the EU Directive 2010/63/EU and the Spanish law RD 53/2013 regarding the protection of experimental animals.

Biosafety and bioavailability studies

Following the experimental feeding period (5 weeks), overnight-fasted rats were euthanized by intracardiac exsanguination under anesthesia with 1.5% isoflurane. Plasma was separated out by centrifugation (10 min at 5000 rpm) using sterile tubes pre-treated with EDTA. The supernatant collected was stored at -20°C until analysis. Plasma levels of total cholesterol (TC), HDL cholesterol, triglycerides, glucose and circulating renal and liver damage biomarkers (creatinine, uric acid, bilirubin, alanine transaminase (ALT), aspartate transaminase (AST)) were measured in duplicate for each sample using a Covas C311 Autoanalyzer (Roche, Basel, Switzerland) specifically calibrated for rodent samples. All enzymatic colorimetric kits and internal quality controls were supplied by Roche (Basel, Switzerland).

Livers, spleen, kidneys and testis or ovaries were collected, weighed and washed in ice-cold PBS. Samples were processed for each tissue, keeping a fraction fixed in 10% neutral buffered formalin for 24 hours and the other part was

immediately frozen in liquid nitrogen and storage at -80 °C until analysis. Fixed samples were embedded in paraffin for further analysis by immunohistochemistry. Afterwards, frozen liver samples were subjected to homogenization to detect eritadenine (see later).

Eritadenine determination by HPLC-DAD

Eritadenine was extracted from samples and quantified following two different methods. One of them (method 1) was based on the procedure of Enman *et al.* (2007) [12] with slight modifications. In this case, samples (1 g) were mixed with 20 mL of 80% methanol (v/v) and stirred in the dark for 3 h. Then, the mixture was filtered through a 14–18 µm pore size paper filter (GE Healthcare Europe GmbH 1240, Barcelona, Spain) and methanol was removed on a rotary vacuum evaporator (60 °C), keeping the sample protected from light. Afterwards, the dried extract was mixed with 10 mL Milli-Q water, washed 3 times with diethyl ether, mixed with 40 mL ethanol (4:1, v/v) and kept overnight at -20 °C. Finally, the sample was filtered through filter paper, and ethanol was removed using the rotary vacuum evaporator and water using a freeze-dryer. The resulting eritadenine extract was stored at -20 °C until further use.

The second extraction method (method 2) followed the Afrin *et al.* (2016)[13] procedure. Briefly, samples (1 g) were mixed with 10 mL of 60% ethanol (v/v) and stirred for 2 min. The mixture was subjected to centrifugation (7000 rpm, 15 min) in a Heraus Multifuge 3SR+ centrifuge (Thermo Fisher Scientific, Madrid, Spain) and the supernatant was carefully collected. The pellet was extracted twice and supernatants were pooled together, filtered and dried as explained in method 1.

Eritadenine was extracted from livers by homogenizing the organs with an IKA Werke T8 Ultra Turrax (Ika Works Inc., Staufen, Germany) at maximum power. Resulting homogenates were mixed with 10 mL of 60% ethanol (v/v) and stirred for 2 min, following method 2.

The identification and quantification of eritadenine were carried out using an HPLC system (Pro-Star 330, Varian, Madrid, Spain) equipped with a PDA detector (Pro-Star 363 module, Varian, Madrid, Spain). Samples were dissolved in the mobile

phase (5 mg/mL), injected (10 µL) into a C18 Spherisorb ODS2 analytical column (4 x 250 mm, 5 µm, Waters, Missisagua, Ontario, Canada) and developed at 0.5 mL/min with water : acetonitrile (98:2, 1% v/v TFA). Eritadenine was quantified at 260 nm using a commercial standard (0.004 – 0.25 mg/mL). The compound eluted after 10.5 min and showed the characteristic eritadenine UV-spectrum.

Statistical analysis

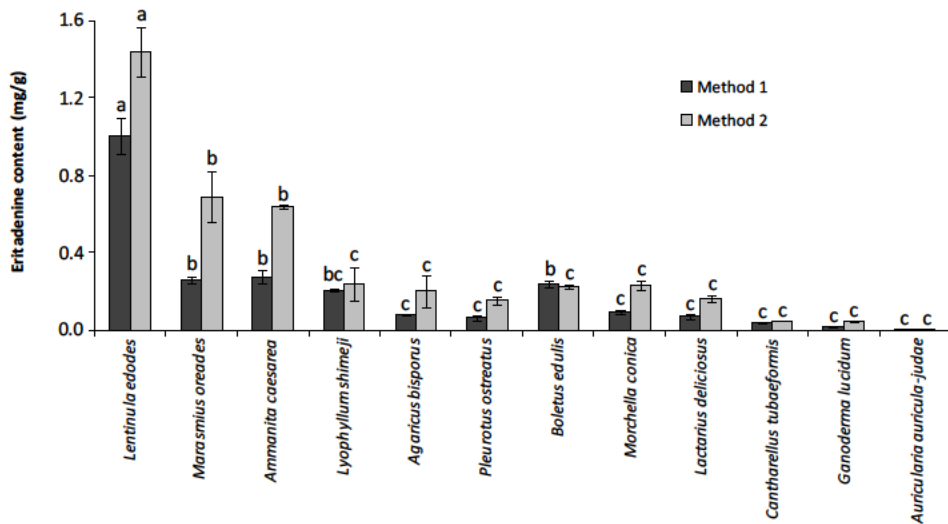
Differences were evaluated at 95% confidence level ($P \leq 0.05$) using one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison test. Statistical analysis was performed using SPSS V.13.0 software (SPSS Institute Inc., Cary, NC) and GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA, USA).

Results and discussion

Determination of eritadenine in several edible mushrooms

Eritadenine was firstly isolated from *L. edodes* [5] but not many other species were investigated to study whether they might contain interesting concentrations. Therefore, before selecting shiitake as a source of the metabolite, a preliminary screening of a few other related mushroom species was carried out. Eritadenine was extracted from selected mushrooms following two already described methods because they both claimed to be specific for eritadenine determination but their procedures were different [12, 13]. Results indicated that indeed *L. edodes* was the mushroom with higher eritadenine concentrations but a few others species contained it too although in lower concentrations (Figure 1a). Extraction of eritadenine using method 1 yielded slightly lower levels of this metabolite than extraction using method 2 and this difference seemed to occur similarly within all the analyzed species. Apparently, the longer incubation times utilized in method 1 did not increase extraction yield.

a)



b)

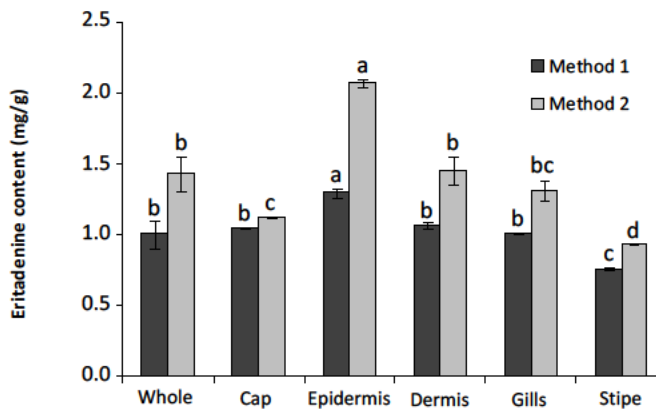


Figure 1. Eritadenine content (mg/g dry weight) in a) several mushroom species and in b) different fruiting body tissues from *Lentinula edodes* determined according to two different methods. Different letters (a-d) denote significant statistical differences between species for the same method ($P \leq 0.05$).

L. edodes contained 1.0 or 1.4 mg/g (depending on the method utilized), levels that were lower than those in some publications [12,13] but higher than others [14]. However, the fact that both methods indicated similar concentrations suggested

that differences with respect to the other publications might be because of different cultivation conditions, processing, mushroom strains, etc. Other mushroom species such as *Marasmius oreades*, belonging to the same family as *L. edodes* (Marasmiaceae), showed 0.7 mg/g eritadenine (according to method 2), only half the shiitake levels, suggesting that closely related species might also be used to synthesize significant amounts of this compound. Eritadenine was also found in other mushrooms belonging to the same order as *L. edodes* (Agaricales) such as *Amanita caesarea* (0.6 mg/g), *Lyophyllum shimeji*, *Agaricus bisporus* and *Pleurotus ostreatus* (approx. 0.2 mg/g). But other mushrooms not so closely related, such as *Boletus edulis*, *Morchella conica* and *Lactarius deliciosus* also contained similar eritadenine concentrations (0.2 mg/g). Therefore, the presence of the compound in higher concentrations might be only attributed to the *Lentinula* genus.

Production of eritadenine by shiitake fruiting bodies

A more detailed study about the eritadenine biosynthesis was carried out by determining its production within different fruiting bodies tissues and during their development. The results indicated that differences between tissues were more pronounced when using method 2 than 1. Within the cap, eritadenine was present in higher concentrations in the epidermis than in the dermis or gills (Figure 1b). The role of this compound in the mushroom metabolism has still not been elucidated although some studies suggested its involvement in defense (as antibiotic nucleoside) like many other derivatives from the secondary metabolism [15,16]. If this was the case, it seemed adequate to concentrate the compound in the tissue with direct contact with the environment. The stipe also contained the compound but in lower concentration being in concordance with previous observations indicated by Saito et al. (1975) [14] where lower levels of eritadenine were found in all the stipes compared to the caps of several shiitake strains.

Moreover, when eritadenine biosynthesis was studied during the development of the fruiting bodies, the results indicated that the metabolite was produced in similar concentrations during their complete growth since no differences were found within developmental stages (Table 2).

Table 2. Eritadenine content (mg/g dry weight) in different developmental stages of *Lentinula edodes* fruiting bodies. No statistical significant differences were found.

Developmental stage	Eritadenine content (mg/g)	
	Method 1	Method 2
Immature	1.23±0.01	1.46±0.03
Intermediate	1.01±0.09	1.44±0.13
Mature	1.23±0.01	1.71±0.02

Although mature mushrooms showed slightly higher eritadenine contents (using method 2) differences with earlier stages were not statistically significant. Therefore, since method 2 was an easier procedure and differences between samples were broader than method 1, method 2 was selected for further eritadenine quantifications.

Effect of traditional culinary treatments on eritadenine stability and bioaccessibility

In order to investigate whether eritadenine might be assimilated by shiitake consumers, the effect of traditional culinary treatments applied to the mushroom before its consumption was evaluated. Furthermore, processed fruiting bodies were submitted to an *in vitro* digestion model mimicking human digestion to determine eritadenine concentrations in the bioaccessible fraction.

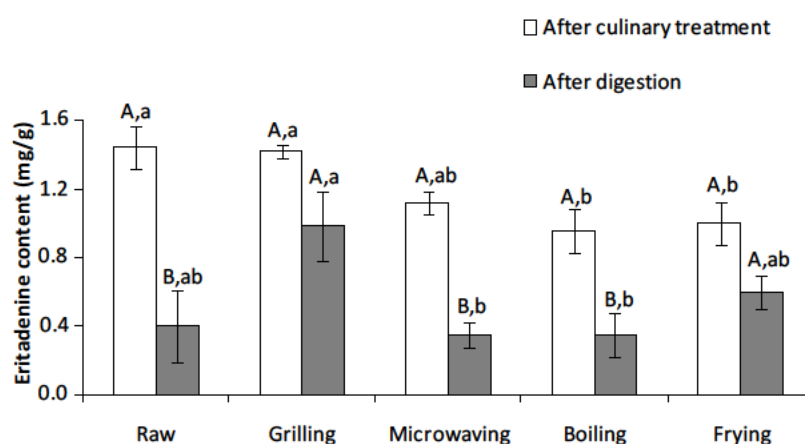
The dry heat irradiated during grilling did not significantly affected eritadenine levels compared to raw mushrooms (Figure 2a). Apparently, eritadenine was heat stable as also indicated by other studies (Chapter 3, Manuscript 3; Chapter 4, Manuscript 1). However, when other cooking treatments involving water as heat transmitter medium were used such as microwave or boiling, eritadenine content was reduced. Temperatures in these processes were milder than grilling; therefore, its losses might be due to lixiviation into the aqueous medium because of its hydrophilic nature. But, when a lipid medium was utilized for frying, a decrease in eritadenine content was also noticed. Perhaps the deeper penetration of the oil through the

mushroom tissues brought the higher reached temperature (160 °C) to more internal parts of the mushroom entering more in direct contact with the molecule. When dry heat is used, although the irradiated temperature is higher than frying (200 °C), only the skin and first outer layers of tissue receive such irradiation. The inner parts are cooked by their own water content (meaning maximum 100 °C) because of the cooling effect of water evaporation and because biological materials show very low thermal conductivities. The dry heat reaching the skin induced a Maillard crust that might impair the leaching out of constitutive water containing the eritadenine.

After the culinary treatments, shiitake mushrooms were treated with digestive enzymes at specific pH values that simulate mastication and stomach and intestinal digestion. The resulting water-soluble digestates were considered as the fraction that might reach the enterocyte layer in the intestine where absorption takes place. The results indicated that, although grilled mushrooms showed similar eritadenine values than raw mushrooms after the digestion, the compound from the grilled mushrooms was more bioaccessible since almost 69.3% of the compound was found in the bioaccessible fraction, while if raw mushrooms were digested only 26.9% was noticed (Figure 2a). Apparently, the heat treatment partially disintegrated the structural fibers and hyphae facilitating its release into the bioaccessible phase. It could be similar to the effect of the glucanases and chitinases utilized by Enman *et al.* (2007) [12] to extract higher eritadenine concentrations from shiitake tissues.

Moreover, if cooking and digestion are considered, the high temperatures reached during frying were not so detrimental because although part of the eritadenine was lost during the process, it facilitated the release of the remaining levels into the bioaccessible fraction (59.1%) reaching levels higher than the other culinary treatments involving water (30.8% bioaccessibility after microwave cooking and 35.4% after boiling).

a)



b)

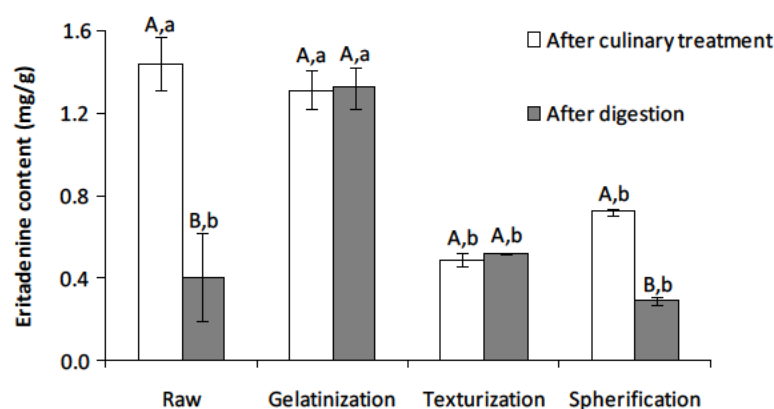


Figure 2. Eritadenine content (mg/g dry weight) in *Lentinula edodes* submitted to different a) traditional and b) modern culinary treatments and after an *in vitro* digestion model. Different letters (a-b) denote significant statistical differences between culinary treatments and (A-B) between the same culinary treatment and after *in vitro* digestion ($P \leq 0.05$).

The further degradation in the latter treatments could be due to the fact that once the eritadenine is leached in the medium after cooking, it might be more

accessible to digestive enzymes. Nevertheless, the eritadenine levels that, in principle, still might reach the enterocytes are similar to those of raw mushrooms.

Effect of modern culinary treatments on eritadenine stability and bioaccessibility

The effect of novel culinary procedures such as thickening (called ‘texturization’ by chefs), spherification and gelatinization on the eritadenine concentrations was also tested. The eritadenine content of gelatinized mushroom powder was similar to the non-treated powder (raw), suggesting that the mild treatment required to prepare this gel did not negatively influenced its levels (Figure 2b). However, when the mushroom powder was subjected to texturization or spherification, a large reduction of eritadenine was noticed. During texturization high temperatures were used to dissolve the agar-agar but spherification was carried out at room temperature; therefore, degradation by heat could not be the reason for their lower eritadenine content. Moreover, the above results indicated that the compound resisted temperatures higher than 100 °C. Thus, the other possibility might be that, during the culinary procedure, eritadenine could be scavenged by the polysaccharides used to elaborate the dishes (alginate and agar-agar) forming complexes that hindered eritadenine release with the extraction method utilized (method 2). The fact that Enman *et al.* (2007) [12] suggested to use glucanases and chitinases for its extraction might also support this possibility, particularly because if the gel was generated using proteins such as gelatin, an easy release of the compound was noticed.

The eritadenine of the shiitake gels generated using proteins or agar-agar was protected from digestive enzymes as its levels remained the same after digestion (Figure 2b). However, when spherification was carried out only 40.1% of the spherified eritadenine was bioaccessible; perhaps, the bonds between alginate and eritadenine were stronger than in the other two gels and were resistant to digestion. Nevertheless, its bioaccessible levels were similar to raw mushroom powder with no culinary treatment.

Biosafety of diets containing eritadenine

The animal intervention was performed with male and female rats administrated two different eritadenine doses (high (HE) and low (LE)) and a control group (C) without the metabolite.

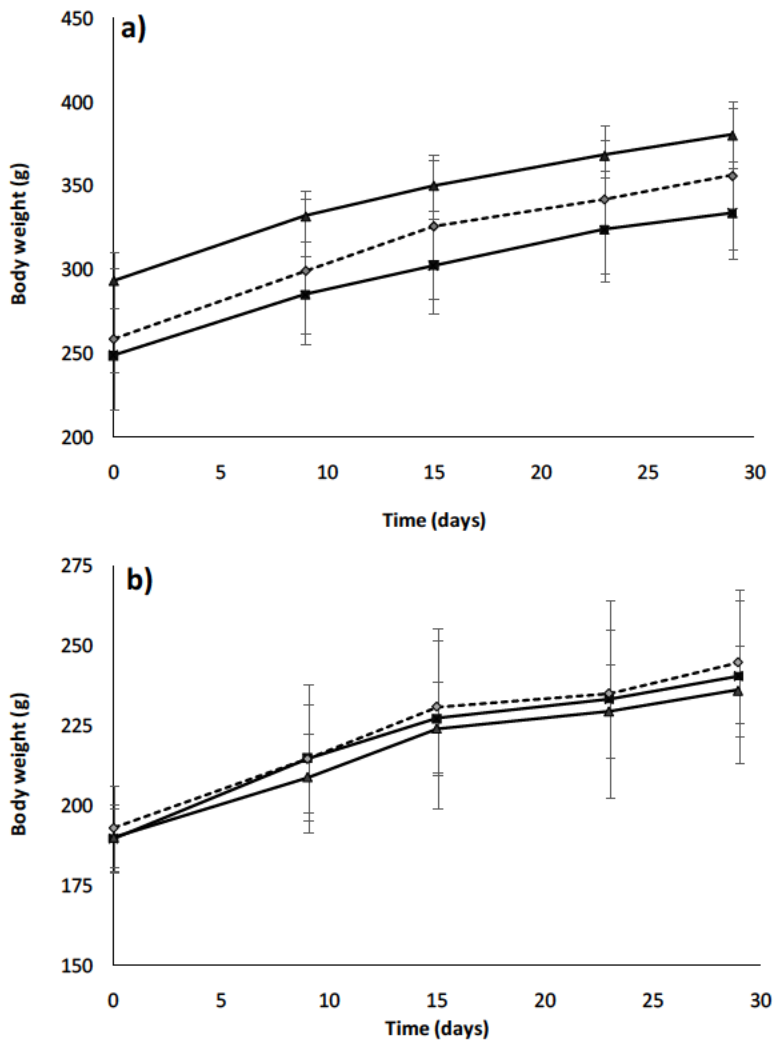


Figure 3. Body weight gain during eritadenine supplementation. a) Male and b) female animals supplemented with high dose (▪) (0.021 mg/g animal /day) or low dose (▲) (0.01 mg/g animal /day) of eritadenine and control group (*). No statistical significant differences were observed between groups.

The LE group received the same eritadenine concentration as previous animal studies since it was indicated as an effective dose with hypocholesterolemic effect in mice [7]. The HE group received double the amount to test not only its effectiveness but also its biosafety.

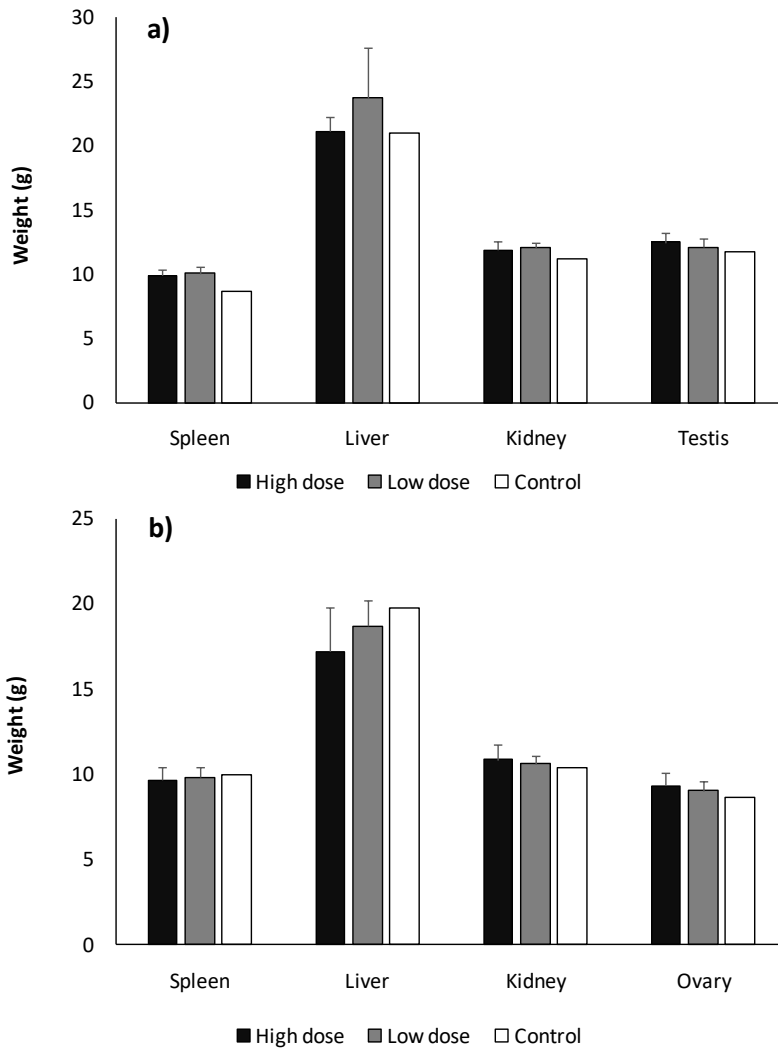


Figure 4. Organ weight after eritadenine supplementation. Total weight of spleen, liver, kidney and testis/ovary of a) male and b) female animals supplemented with high dose (black bars, 0.021 mg/g animal /day) or low dose (grey bars 0.01 mg/g animal /day) of eritadenine and control group (white bars). No statistical significant differences were observed between supplemented groups.

However, the compound was administrated as a food-grade eritadenine-enriched extract to study its potential as a functional ingredient to formulate novel foods and determine whether the rest of the compounds included in the preparation interfered with or enhanced its bioavailability. During the whole intervention, the appearance, behavior and physiology of animals remained stable. Animal weight gain was recorded during the whole intervention and no differences were observed between groups (Figure 3).

Independently of the studied sex, tissues collected and evaluated did not show damages or differences in weight compared with the control group (Figure 4). Based on those results, further immunohistochemical analyses were not performed. Plasma samples were determined to evaluate circulating lipid profile, glucose and biomarkers related to renal and liver function (Table 3). All circulating biomarkers determined were not different from those reported in the control group, except for AST concentrations (aspartate transaminase) that were significantly lower in the LE group than that in the C group (males and females) and total cholesterol that was significantly lower in the LE group than in the C group but only in males. But a slight increase of uric acid levels was also noticed with increasing extract administration.

Bioavailability and hypocholesterolemic properties of eritadenine

Eritadenine could be detected in livers using the previously described chromatographic method (Figure 5). Control rats did not show the eritadenine peak with R.T. 10.5 min while a compound with spectrum compatible with eritadenine could be detected in some of the livers treated with both doses. In 3 livers from the male rats treated with high eritadenine concentration, the molecule was present within 262.9 to 107.1 $\mu\text{g/g}$ fw while only 2 of those treated with lower dose showed eritadenine (41.9 and 101.3 $\mu\text{g/g}$). In the liver from female rats, concentrations were slightly lower, being detected only in 2 of the livers from rats administrated with the higher dose (77.4 and 89.6 $\mu\text{g/g}$) and only 1 of the livers administrated with the lower dose (67.5 $\mu\text{g/g}$).

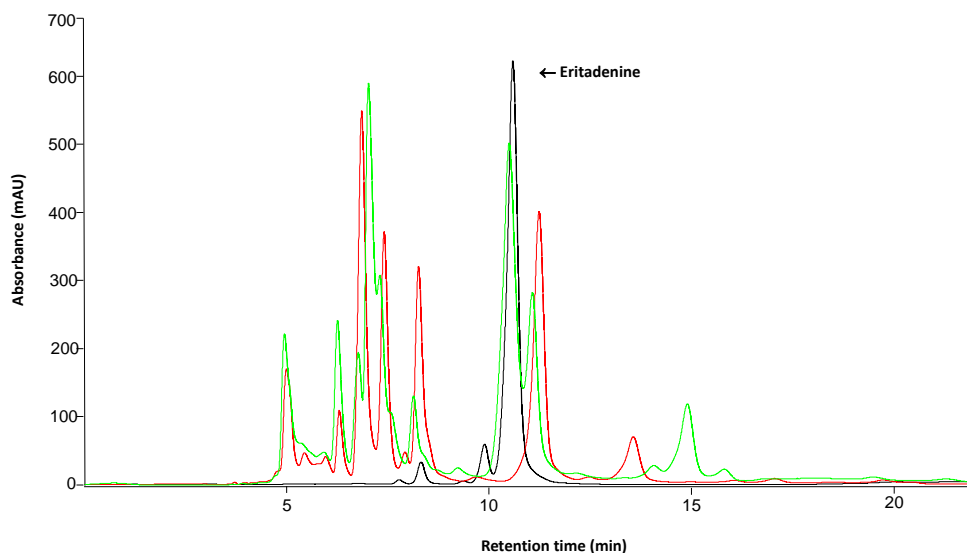


Figure 5. Chromatograms of liver homogenates obtained from rats supplemented with none (red line) or high (green line) eritadenine dose and an eritadenine standard (black line).

Table 3. Plasma lipid profile, glucose concentrations and circulating liver and renal damage biomarkers (all values are expressed in mg/dL except for ALT and AST that are IU/L) in male rats. Statistical significant differences were indicated with the symbol (*).

Males	High dose	Low dose	Control
Total cholesterol (TC)	60.75±2.75	52.75±17.06*	80.00±11.50
HDL-cholesterol	23.25±1.63	18.13±5.45	20.95±2.05
TC/HDL	2.62±0.09	2.89±0.10	3.80±0.25*
Triglycerides	100.00±33.46	71.50±10.50	109.50±19.02
Glucose	105.50±14.15	85.25±3.30	78.50±16.38
Creatinine	0.29 ±0.01	0.33±0.06	0.33±0.06
Uric acid	53.75±9.61	41.75±2.22	43.00±5.72
Bilirubin	0.05±0.02	0.07±0.03	0.04±0.03
ALT	38.50 ±8.19	34.25±2.63	33.00±4.32
AST	98.25 ±9.74	95.00±12.68*	129.00±14.73

Moreover, an approx. 24% lowering of the atherogenic index TC/HDL was noticed with the lower eritadenine concentration tested compared to the controls (Tables 3 and 4).

Table 4. Plasma lipid profile, glucose concentrations and circulating liver and renal damage biomarkers (all values are expressed in mg/dL except for ALT and AST that are IU/L) in female rats. Statistical significant differences were indicated with the symbol (*).

Females	High dose	Low dose	Control
Total cholesterol (TC)	71.50±11.73	77.25±9.50	87.00±2.16
HDL-cholesterol	21.77±3.03	23.45±3.14	20.20±1.66
TC/HDL	3.27±0.12	3.30±0.05	4.33±0.35*
Triglycerides	70.75±28.25	86.50±19.82	103.00±30.74
Glucose	84.25±9.54	101.00±14.49	87.00±9.31
Creatinine	0.31±0.02	0.31±0.03	0.35±0.04
Uric acid	51.25±9.61	47.00±4.55	43.25±3.59
Bilirubin	0.02±0.02	0.05±0.04	0.02±0.01
ALT	43.50 ±15.80	34.50±9.75	32.75±9.39
AST	98.50 ±7.23	86.25±11.15*	119.75±16.88

This reduction was slightly less pronounced than the approx. 29% noticed in mice for the same eritadenine concentration [7]. Differences could be due to the different animal/physiological conditions (mice were hypercholesterolemic) or because eritadenine was administered as a standard compound in the previous study. The latter case would indicate that the use of a fungal extract partially impaired the eritadenine bioavailability. Nevertheless, in the same study, *L. edodes* was also directly administered and effective lowering of cholesterol levels was reached (5 and 10% *L. edodes* administration induced, respectively, 10 and 39 % TC/HDL reduction). Similar values were obtained in this study for male rats (24 and 31 % reduction was noticed for the tested doses, administered as 4.8 and 10 % of the diet). Unfortunately, the previous study did not determine the precise eritadenine concentration in the mushroom, nor indicated the animal sex, so no further comparison could be made.

Conclusions

Besides *Lentinula edodes*, eritadenine was found in significant amounts in other mushrooms such as *Marasmius oreades* and *Amanita caesarea*. It was synthesized during the complete fruiting body growth, being present in higher concentrations in the skin. The molecule showed certain thermal stability since losses due to common culinary treatments were lower than 35% in all cases. Grilling was recommended more than other methods *e.g.* boiling or microwaving not only to maintain high eritadenine concentrations but because it also enhanced its bioaccessibility, probably by facilitating extraction of the compound from the food matrix or protecting it from digestive enzymes. Moreover, a careful selection of the food additives utilized in molecular gastronomy should be made since the use of hydrocolloids such as alginates or agar-agar might scavenge the compound impairing its bioaccessibility. Administration of eritadenine to rats, supplemented as a bioactive ingredient in a normal diet, did not induce damage to any organ or metabolic disorder when added at concentrations up to 21 mg per kg animal per day for 35 days; therefore, it could be considered as safe. It was detected in liver, suggesting that it was bioavailable and could reach that tissue. The extract reduced TC/HDL index in serum of male/female rats although it slightly increased uric acid levels. Therefore, functionalization of foods including an eritadenine-enriched extract obtained from shiitake mushrooms to design a hypocholesterolemic product could be possible since apparently it resists culinary processing, it was not toxic, and it was absorbed and was effective at lowering cholesterol in rats. However, clinical trials are still necessary to confirm these effects in humans.

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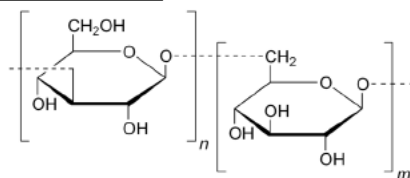
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Chapter 3

Production of β -D-glucan-enriched extracts



Preface

Fungal β -D-glucans, particularly those from *Lentinula edodes*, are the most studied polysaccharides because of their hypocholesterolemic, antioxidant, antitumoral, antimicrobial, prebiotic, immunomodulatory, anti-inflammatory, etc. properties. However, their many activities differed depending on the report, partially because of the different extraction procedures followed to obtain β -D-glucans-enriched fractions from mushrooms. Conventional methods might be too gentle and not all the polysaccharides are extracted or they might be aggressive inducing partial degradation or destruction of native 3D conformation. Therefore, specific advanced technologies were investigated as alternative and more environmentally-friendly methods to obtain bioactive polysaccharides. In this chapter, microwave- (MAE) and ultrasound-assisted (UAE) extractions, subcritical water-extractions (SWE) and even pretreatment with supercritical CO₂ (SFE) and combinations of the mentioned technologies were tested.

The work entitled *Strengths and weaknesses of the aniline-blue method used to test mushroom (1 \rightarrow 3)- β -D-glucans obtained by microwave-assisted extractions* describes an experimental design that was carried out to define the best conditions to obtain polysaccharide-enriched fractions from *L. edodes* using MAE. When the (1 \rightarrow 3)- β -D-glucans present in the extracts were quantified using a reported fluorimetric method, results suggested that fluorimetric determinations might be under- or over-estimating their amounts depending on the standard utilized and the mushroom species studied and therefore, the precise extract composition was also confirmed by GC-MS and NMR analysis.

The use of UAE and SWE as advanced extraction technologies was studied in the work named *Testing the effect of combining innovative extraction technologies on the biological activities of obtained β -D-glucan-enriched fractions from Lentinula edodes* where those methods, their combinations and a pretreatment with SFE were compared with conventional protocols to extract bioactive polysaccharides from shiitake mushrooms. The obtained extracts were firstly analysed by standardized colorimetric, enzymatic and fluorimetric methods to quantify their total carbohydrates, total polysaccharides, β -D-glucans (distinguishing between (1 \rightarrow 3)-

and (1→3),(1→6)-linkages) and chitins contents, but also by more specific analytical tools such as NMR, HPSEC and GC-MS to confirm the nature of their constituents. Moreover, the effect of the extraction using these technologies was studied on 2 of the biological activities recorded for the obtained extracts (on their HMGCR inhibitory activities and on their ability to modulate pro-inflammatory cytokine secretion in LPS-activated THP-1/M cells).

However, another more easily scalable extraction method to obtain β -D-glucans was also studied leading to the work *Production of a β -D-glucan-rich extract from shiitake mushrooms (*Lentinula edodes*) by extraction/microfiltration/reverse osmosis (nanofiltration) process*. It describes a pilot-scale process to generate large amounts of β -D-glucans-enriched fractions using a solid-liquid extractor coupled to a filtration device with cross-flow membranes (microfiltration, reverse osmosis and nanofiltration membranes). Moreover, a specific mixture (called BGE) was prepared by combining some of the obtained β -D-glucan-enriched fractions with others including eritadenine and other interesting polysaccharides. Since this final BGE mixture showed hypocholesterolemic activities according to the animal study indicated in chapter 2, the protocol was repeated several times to produce the BGE mixture in large quantities for a clinical trial (Chapter 5).

Besides the fractions used for the BGE mixture, the latter extraction method also yielded particular fractions interesting to be studied because of their biological activities. Therefore, the work *Isolation and comparison of α - and β -D-glucans from shiitake mushrooms (*Lentinula edodes*) with different biological activities* reports the further purification of three glucan fractions that contained two completely isolated glucans (a branched (1→3),(1→6)- β -D-glucan and a linear (1→3)- α -D-glucan) and a linear (1→6)- β -D-glucan-enriched fraction with a few interesting biological activities but particularly with cytotoxic activity for tumoral breast cells, innocuous for normal breast cells suggesting potential antitumoral effect.

Manuscript 1

Strengths and weaknesses of the aniline-blue method used to test mushroom (1→3)- β -D-glucans obtained by microwave-assisted extractions

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Abstract

The parameters to extract polysaccharide-enriched fractions (PEF) from mushrooms using MAE (microwave-assisted extraction) were adjusted following a full factorial 3^2 experimental design. The highest yield and total carbohydrate values, using *Lentinula edodes* as model mushroom, were obtained at 180 °C and 30 min. Several mushroom species were submitted to MAE and their PEF yields ranged between 12.1-44.2%. (1→3)- β -D-Glucans determination using a conventional fluorimetric method changed depending on the standard utilized. NMR analyses of PEF indicated that the presence of other polysaccharides in the extracts or their specific folding might impair the proper determination of (1→3) linkages by the fluorophore. Mushrooms from Cantharellales order contained (1→3)- β -D-glucans but they were not detected with the fluorimetric method. Therefore, although the method (after adjustments) was sensitive enough to detect their presence in many mushroom extracts, it cannot be used for all species and it is also not recommended for quantitative determinations.

Introduction

Mushroom polysaccharides showed many beneficial properties for human health. They were described as immunomodulatory, antibacterial, antidiabetic, anti-inflammatory, hypocholesterolemic agents [1]. Although mushrooms can synthesize a wide variety of polysaccharides, β -D-glucans are those pointed as responsible for most of the biological activities. β -D-Glucans are present in all mushroom species since they are the major constituents of fungal cell walls contributing to their structure [2]. Although linear β -D-glucans were also isolated from certain species, their molecular structures mainly contain a (1 \rightarrow 3)-linked backbone chain with substitutions at O-6 by single units of β -D-glucopyranose [2]. Previous studies suggested that the biological properties of these compounds are related to their chemical structure. Thus, characteristics such as the linkage type, polymerization level [3] or degree of branching [4] of such molecules might determine not only their biological but also their physico-chemical properties (e.g. solubility) [4-6]. Zhang et al. (2005) [7] reported that the complexity of the helical conformation (i.e. single or triple) determined the ability of the (1 \rightarrow 3),(1 \rightarrow 6)- β -D-glucans to inhibit the tumour growth [8]. In addition, modified pachymaran, a (1 \rightarrow 3),(1 \rightarrow 6)- β -D-glucan, also showed antitumor properties, after β -(1 \rightarrow 6) side chain removal [3]. These evidences indicated that preserving the structural integrity of fungal β -D-glucans (mostly (1 \rightarrow 3),(1 \rightarrow 6)- β -D-glucans) during the extraction and analysis processes, might be essential to maintain intact their bioactivities.

Protocols including hot water extractions are the most common methods to obtain fungal polysaccharides [2,3]. However, polysaccharides with complex conformations require more aggressive extraction methods such as hot alkali solutions and the use of chloroacetic or concentrated sulphuric acids, but they might also induce undesirable structural modifications [9, 10, 11]. Alternatively, new extraction technologies using water such as pressurized liquid extraction (PLE), ultrasound assisted extraction (UAE) or microwave assisted extraction (MAE) were also studied as effective tools for harmless polysaccharide extractions [12]. MAE uses microwaves energy to heat directly the extraction solvent without a heat transfer from the vessels to the solvent. It maintains thermal gradients to minima inducing a more

homogeneous temperature through the sample [10]. The high pressure generated during the extraction process maintains water in its liquid state at temperatures higher than its boiling point, accelerating mass transfer of compounds [2, 13]. Several studies used MAE to extract total polysaccharides from mushrooms [14, 15], however, only a few studied the β -D-glucans content of the obtained extracts [2, 16].

A common method used to study β -D-glucans is based on the sirofluor ability to bind polysaccharides with (1 \rightarrow 3)- β -D-glucan branches [9, 17, 18]. Some reports claimed that such fluorimetric method is selective for (1 \rightarrow 3)- β -D-glucan binding but, it showed certain restrictions that were not always considered, for instance, the polymerization degree, presence/absence of substituents and their tridimensional conformation might modulate the fluorimetric determination [2, 17].

Therefore, a three level factorial experimental design was carried out to find the most suitable MAE conditions to obtain β -D-glucans-enriched extracts using *Lentinula edodes* as mushroom model. Afterwards, a screening of other species was performed using similar extraction conditions. The influence of the (1 \rightarrow 3)- β -D-glucan structure on the fluorimetric assay was studied (using also NMR) to remark its limitations and the method was adjusted to improve its accuracy for the detection of β -D-glucans in MAE extracts.

Materials and methods

Biological material, reagents and standard compounds

Powdered *Lentinula edodes* S. (Berkeley) fruiting bodies with a particle size lower than 0.5 mm and a moisture content less than 5% were purchased from Glucanfeed S.L. (La Rioja, Spain). Dried fruiting bodies, commercially available in local markets, from *Auricularia auricula-judae* (Bull. Ex St.Amans) Berck,, *Cantharellus cibarius* (Fr.) *Cantharellus tubaeformis* (Fr.), *Cantharellus lutescens* (Herve.) Fr., *Cantharellus cornucopoides* (L. Ex Fr.) Pers., *Boletus edulis* (Bull. Ex Fr.), *Lactarius deliciosus* (Fr.), *Pleurotus pulmonarius* (Fr.) Quel., *Pleurotus eryngii* (D.C. Ex Fr.) Quel, *Morchella conica* (Pers.), *Agrocybe aegerita* (Briganti) Singer, *Amanita caesarea* (Scop. Ex Fri.) Pers. Ex Schw. and *Hypsizygus marmoreus* (Peck)

H.E. Bigelow were purchased and ground using a Grindomix GM200 Retsch mill (VERDER Group, The Netherlands) as described by Ramirez-Anguiano et al. (2007) [19]. Powdered mushrooms (MP) were stored at -20 °C under darkness until further use.

Absolute ethanol was purchased from Panreac (Barcelona, Spain) as well as concentrated sulfuric acid. Phenol, sodium borohydride, sodium hydroxide pellets, glycine, aniline blue diammonium salt 95% and hydrochloride acid 37% were obtained from Sigma-Aldrich (Madrid, Spain). Compounds used as standards were two linear β -D-glucans: curdlan (a (1 \rightarrow 3)- β -D-glucan from *Alcaligenes faecalis*, Sigma-Aldrich, Madrid, Spain) and a (1 \rightarrow 6)- β -D-glucan isolated from *A. bisporus* (named B6G) [20]; and two branched (1 \rightarrow 3),(1 \rightarrow 6)- β -D-glucans: schizophyllan from Contipro Biotech (Dolní Dobruška, Czech Republic) and a chemically characterized glucan (PUL, previously named B1316PP) extracted from *P. pulmonarius* [21]. Moreover, a heteropolysaccharide (mannogalactan, MG) isolated from *P. pulmonarius* [21] and commonly found in Basidiomycetes, was also used to compare with the β -D-glucans.

Microwave-assisted extractions (MAE)

Polysaccharides-enriched fractions (PEFs) were obtained using an automated microwave extraction system coupled to a MAS 24 auto-sampler (Monowave 300, Anton Paar GmbH, Graz, Austria). Extractions were performed at 1:30 mushroom powder:water ratio, 850W power, 2455 MHz frequency and 30 bar pressure as was described by Smiderle et al. (2017) [12]. After MAE, the samples were centrifuged and the pellet discarded. An aliquot of the supernatant (200 μ L) was used to estimate the total carbohydrate content (TCH) in the MAE extracts. Afterwards, the polysaccharides were precipitated with ethanol under cold conditions following the procedure of Smiderle et al. (2017) [12]. Obtained polysaccharide-enriched fractions (PEF) were collected, freeze-dried, weighted and kept at -20 °C until further use (Equation 1, Figure 1).

$$(1) \text{ PEF yield (\%)} = \frac{\text{weight of polysaccharide enriched fraction after MAE precipitation (g)}}{\text{weight of mushroom powder (g)}} \times 100$$

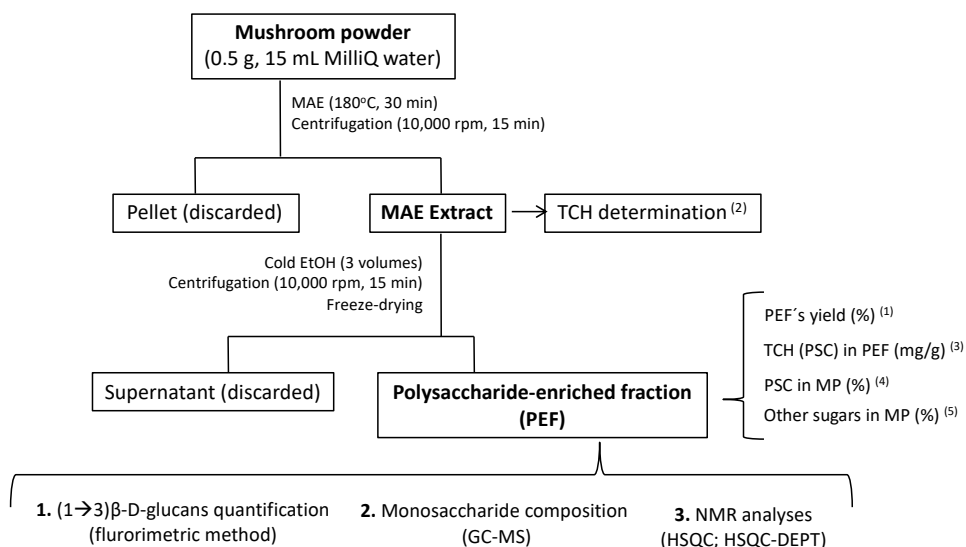


Figure 1. Workflow of the experiments developed in this work. Superscripts 1-5 correspond to the specific equations used to determine the corresponding values.

Design of experiment (DoE) approach for MAE of *Lentinula edodes*

In order to explore the efficiency of MAE to obtain PEFs, a full factorial three level experimental design (3^k) was selected using *L. edodes* as mushroom model. Two factors (k), extraction temperature and time, were studied and their ranges were set according to the equipment limitations and results reported for other mushroom species (respectively 50 – 180 °C and 5 - 30 min) [12]. PEF yield (% , g PEF/ 100 g MP) and TCH (mg equivalents of glucose/ g MP) were selected as response variables. Eleven randomized extractions were performed following the parameters indicated in Table 1, i.e. three levels per factor (3^2) with two additional central points. Most convenient MAE conditions achieved for *L. edodes* were selected to obtain PEFs from the other mushroom species.

Table 1. Full factorial 3² experimental design for *L. edodes* MAE. ¹TCH, total carbohydrates content in MAE extracts; ²mg equiv. Glc/g MP, milligrams of equivalent glucose per gram of mushroom powder. ³PEF yield, polysaccharide yield of the enriched fraction (PEF) obtained from MAE extracts.

Exp. Number	Factors		Response variables	
	Temperature (°C)	Time (min)	TCH in MAE ¹ extracts (mg equiv. Glc/g MP) ²	PEF yield ³ % (w/w)
1	180	5	263.21 ± 18.4	15
2	50	5	107.52 ± 5.8	4.6
3	115	30	88.62 ± 3.12	4.4
4	50	17.5	107.36 ± 8.5	4
5	115	17.5	83.33 ± 2.0	4
6	115	5	98.65 ± 31.0	5
7	115	17.5	86.21 ± 31.4	3.4
8	50	30	116.80 ± 4.9	2.8
9	180	30	290.43 ± 20.6	15.4
10	180	17.5	238.76 ± 12.5	16.4
11	115	17.5	80.38 ± 19.2	3.6

Determination of carbohydrates from MAE extracts

The total carbohydrate content (TCH) in MAE extracts and PEFs (obtained after precipitation of MAE extracts) (Figure 1) was measured using the phenol-sulfuric acid method, as detailed by Smiderle et al. (2017) [12]. Since only traces of monosaccharides or oligosaccharides might remain in obtained PEFs, in this case, the TCH values indicated the total polysaccharide (PSC) concentration. Calculations were performed as follow (including unit conversions):

$$(2) \text{ TCH in MAE extracts (mg/g)} = \frac{\text{equivalents of glucose in MAE extracts (mg)}}{\text{weight of MP (g)}}$$

$$(3) \text{ TCH (or total PSC) in PEFs (mg/g)} = \frac{\text{equivalents of glucose in PEF (mg)}}{\text{weight of PEF (g)}}$$

$$(4) \text{ PSC in MP after MAE ppt. (\%)} = \frac{\frac{\text{equivalents of glucose in PEF (mg)}}{\text{weight of PEF (g)}} \times \frac{\text{weight of PEF (g)}}{\text{weight of MP (g)}}}{10 \text{ (unit conversion)}}$$

$$(5) \text{ Other sugars in MP after MAE ppt. (\%)} = \frac{\text{TCH in MAE extracts}}{10 \text{ (unit conversion)}} - \text{PSC in MP by MAE precipitation}$$

Supernatants obtained after MAE (Equation 2, Figure 1) were diluted for all species as 1:15 (extract: water) except for *L. edodes* (1:5) and for *A. auricula-judae* and *P. eryngii* (1:30). TCH in PEFs (Equation 3, Figure 1) were diluted for all species as 1:5. Samples were analyzed in triplicate and glucose was used as standard.

Determination of (1→3)- β -D-glucans from PEF

β -D-Glucans were determined by the fluorimetric method firstly described by Evans et al. [17]. This method uses an impurity from the aniline blue stain (sirofluor) as fluorochrome because of its ability to bind polysaccharides with (1→3)- β -linkages (Figure 1). Thus, the presence of (1→3)- β -D-glucans was determined in freeze-dried MAE polysaccharide fractions (PEF) using the method reported by Ko & Lin (2004) [18] with some modifications concerning the sample preparation and the analytical procedure. Briefly, fractions were solubilized in 0.05 M NaOH with 1% NaBH₄ (0.02 mg/mL) to preserve the polysaccharides integrity. Then, samples (300 μ L) were mixed with 30 μ L of 6 M NaOH and 630 μ L of a dye solution (0.1% aniline: 1 M HCl: 1 M glycine/NaOH buffer pH 9.5 33:18:49, v/v/v) and incubated at 50 °C for 30 min in a water bath. Each mixture (250 μ L) was transferred to a 96-well plate and analyzed using a M200 Plate Reader (Tecan, Mannedorf, Switzerland) with excitation and emission wavelengths of 398 nm and 502 nm respectively. The buffer was freshly prepared before use to avoid degradation.

Fluorescence signals relative to the β -D-glucans content in PEF were obtained at 0.002, 0.01 and 0.02 mg/mL for all previously mentioned species. The standards for β -D-glucans were used in a range of 0-0.02 mg/mL for calibration curves and a solution of 0.05 M NaOH with 1% NaBH₄ was used as blank. Determinations were carried out in triplicate. The selection of the adequate standard for the detection of (1 → 3)- β -D-glucans in a mushroom extract was dependent on recorded fluorescence slopes.

Analysis of monosaccharide composition by GC-MS

The polysaccharide-enriched fractions (PEF) from MAE extracts (1 mg) were hydrolyzed with 2M TFA at 100 °C for 8 h, followed by evaporation to dryness

(Figure 1). The dried carbohydrate samples were dissolved in distilled water (100 μL) and 1 mg NaBH_4 was added. The solution was held at room temperature overnight to reduce aldoses into alditols [22]. The product was dried, the excess of NaBH_4 neutralized by the addition of acetic acid and removed with methanol (x2) under a compressed air stream. Alditols acetylation was performed in pyridine– Ac_2O (200 μL ; 1:1, v/v), for 30 min at 100 $^\circ\text{C}$. The pyridine was removed by washing with 5% CuSO_4 solution and the resulting alditol acetates were extracted with CHCl_3 . The resulting derivatives were analyzed by GC-MS (Varian CP-3800 gas chromatograph coupled to an Ion-Trap 4000 mass spectrometer), using a VF5 column (30 m x 0.25 mm i.d.) programmed from 100 to 280 $^\circ\text{C}$ at 10 $^\circ\text{C}/\text{min}$, with He as carrier gas. The obtained monosaccharides were identified by their typical retention time compared to commercially available standards. Results were expressed as mol%, calculated according to Pettolino et al. (2012) [23].

Nuclear magnetic resonance spectroscopy

NMR spectra (HSQC and HSQC-DEPT) from PEFs (Figure 1) were obtained using a 400 MHz Bruker model Avance III spectrometer with a 5 mm inverse probe. The analyses were performed at 70 $^\circ\text{C}$ and the samples (30 mg) were dissolved in D_2O (400 μL). Chemical shifts are expressed in ppm (δ) relative to resonance of acetone at δ 30.2 and 2.22 corresponding to ^{13}C and ^1H signals, respectively. NMR signals were assigned on the basis of 2D NMR experiment (HSQC) and literature data.

Statistical analysis

In order to set the experimental conditions and detect the optimal MAE parameters, analysis of variance (two-way ANOVA test) was carried out using the Statgraphics Centurion XVI software (Statpoint Technologies, Warrenton, Virginia, USA). The confidence level was set at 95% ($P < 0.05$) for all cases.

Results and discussion

Response surface study of microwave assisted extractions from *L. edodes*

Powdered fruiting bodies from *L. edodes* were submitted to MAE following a full factorial 3^2 experimental design. Two factors, extraction time and temperature, were tested to estimate the more convenient combination to obtain high TCH values and PEF yields (Table 1). Similar trends in the response surface plots for both variables were obtained (Figure 2), higher TCH and yields were reached at the highest tested temperatures. The model showed an adequate fitting to experimental data supported by the elevated variability percentage in TCH and yield explained by the model (99.4 and 98.8% respectively). The regression equations fitting to the data were:

$$y_1 = 13.1 - 0.22x_1 - 0.12x_2 + 1.3 \cdot 10^{-3}x_1^2 + 6.7 \cdot 10^{-4}x_1x_2 + 6.0 \cdot 10^{-4}x_2^2$$

$$y_2 = 281.08 - 4.12x_1 - 3.89x_2 + 0.02x_1^2 + 5.5 \cdot 10^{-3}x_1x_2 + 0.1x_2^2$$

where the linear and quadratic effects of both factors (x_1 = temperature and x_2 = time) as well as the interaction between them were included for y_1 (yield) and y_2 (TCH) prediction. Only *linear* and *temperature* quadratic terms showed a statistically significant influence on the TCH and yield, following the analysis of the variance results (ANOVA, $p < 0.05$). The factor *time* as well as the interaction between both factors, were defined as statistically insignificant. However, according to the Pareto charts for each variable response (Supplementary Figure 1), the term *quadratic time* affected more to TCH content than to the yield. This effect can be easily visualized in the respective response surface plotting by the curvature along time (Figure 2a).

The temperature and time values that maximize the TCH and PEF yields of MAEs, within the selected conditions ranges, were 180 °C and 30 min. These values were similar to those previously described for other mushroom species such as *Pleurotus ostreatus* and *Ganoderma lucidum* after a similar extraction procedure [12]. The desirable linear regression obtained by the plot observed vs. predicted values for each variable response validated the model (Supplementary Figure 2). Nevertheless,

to verify it, three additional microwave-assisted extractions from *L. edodes* were performed at 180 °C and 30 min. Only slightly lower values were observed for TCH (224.4 ± 6.1 mg equiv. Glc/g MP) and higher values for PEF yield ($19.1 \pm 0.3\%$, w/w) were noticed compared to the software predicted values (respectively 278 mg equiv. Glc/g and 15% w/w). Therefore, the predictive model fitted the experimental behavior.

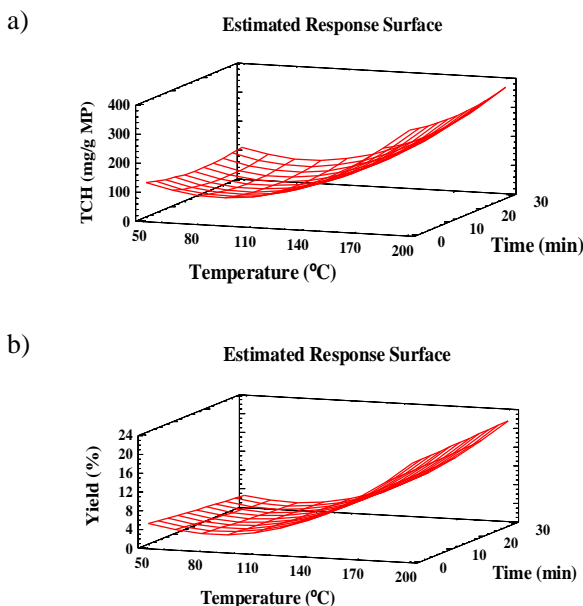


Figure 2. Estimated response surfaces plots for a) TCH content and b) PEF yields after MAE from *L. edodes*.

Substantially higher PEF yields were observed after MAE compared to the recoveries previously reported for conventional hot water extractions, i.e. 5.3% were obtained after seven extractions at 100 °C for 200 min [24]. The higher MAE efficiency could be explained by the more effective disruption of analytes-matrix interactions and an improved mass transference at high temperatures and pressures, resulting in larger recoveries [25, 26]. However, their higher solubilization might also indicate that MAE could generate some physical changes in the tertiary conformations of the polysaccharides enhancing their extractability [3] and/or that some chemical changes such as partial hydrolysis might occur as indicated by other

studies [27, 28]. Nevertheless, since the extraction yield steadily increased and no drastic changes were observed/predicted at least up to 180 °C, the latter possibility seemed unlikely.

Microwave-assisted extractions from other edible mushrooms

MAE were also carried out at 180 °C and during 30 min for the other selected species since with these parameters, the highest TCH and PEF yields were obtained for *L. edodes* and, according to Smiderle et al. (2017) [12], they were also appropriate for other two mushrooms. The yields of obtained PEFs from the selected species ranged between 12.1 (*Cantharellus cibarius*) and 19.1% (*L. edodes*) except for *Auricularia-auricula judae* that showed extremely high yields (Table 2). However, the MAE extracts that showed higher TCH values besides *A. judae* were *Pleurotus eryngii*, *L. edodes* and *Boletus edulis* indicating that the latter two species contain higher levels of monosaccharides and oligosaccharides than the rest of species. In the case of *A. judae*, results indicated that almost half of the dry matter from the mushroom powder could be extracted using MAE obtaining PEFs with 96.4% polysaccharides. *L. edodes* yields were in the range of the previously observed values (15.4%) as also noticed for the other species i.e. *Armillaria luteovirens* (8.40 - 8.34%) [14], *G. lucidum* (11.2%) [12], *Fomitopsis ulmaria* (8.36%) [29], etc. Other mushrooms such as *Morchella conica* showed PEF yields (16.5%) slightly higher than other MAE obtained extracts previously reported (5.86%) [30] and *Pleurotus pulmonarius* showed lower values than other related species such as *P. ostreatus* (32.4%) [12]. However, the different extraction conditions such as shorter times and/or different mushroom varieties or cultivation conditions, might be the reason for the noticed differences.

The TCH content determined in PEFs indicated their polysaccharides concentration (since only traces of monosaccharides or oligosaccharides might be present after the precipitation procedure). Results indicated that, indeed, PEFs were fractions with high polysaccharide content except for *Craterellus cornucopioides* (40.3%). PEF obtained from mushrooms such as *Amanita caesarea*, *Agrocybe aegerita* or *Hypsizygus marmoreus* contained 63.6 to 68.2% PSCs. The rest of the PEF weight might be proteins (perhaps bound to polysaccharides as glucoproteins or

proteoglucans) as some of them might also precipitate with the ethanol concentrations utilized.

Table 2. Total carbohydrate (TCH) in MAE extracts (mg/g), yields of obtained polysaccharide-enriched fraction (PEF) after MAE precipitation, TCH (mg/g) in PEF, PSC (%) in PEF and PSC (%), as well as other sugars, after MAE precipitation. *n=3 independent colorimetric measurements for TCH determination, † n=2 independent MAE extractions.

Species	TCH in MAE extracts (mg/g)*	PEF yield % (w/w)†	TCH in PEF (mg/g)*	PSC in PEFs (%)	PSC in MP after MAE precipitation (%)	Other sugars in MP after MAE precipitation (%)
<i>A. judae</i>	485.5 ± 11.0	44.2 ± 1.3	964.6 ± 65.5	96.4	42.64	5.91
<i>A. aegerita</i>	175.0 ± 3.9	17.6 ± 0.3	682.7 ± 42.5	68.2	12.02	5.48
<i>A. caesaria</i>	180.6 ± 5.5	13.9 ± 1.2	636.4 ± 37.2	63.3	8.85	9.21
<i>B. edulis</i>	226.9 ± 12.8	12.3 ± 0.1	588.8 ± 8.8	58.8	7.24	15.45
<i>C. cibarius</i>	140.3 ± 31.0	12.1 ± 0.3	567.5 ± 49.6	56.7	6.87	7.16
<i>C. cornucopioides</i>	87.19 ± 7.9	15.3 ± 0.6	403.4 ± 1.7	40.3	6.17	2.55
<i>C. lutescens</i>	157.4 ± 18.9	15.7 ± 0.1	608.8 ± 40.7	60.8	9.56	6.18
<i>C. tubaeformis</i>	91.3 ± 3.4	16.4 ± 0.1	511.1 ± 34.8	51.1	8.38	0.75
<i>H. marmoreus</i>	184.8 ± 1.0	12.8 ± 1.4	681.5 ± 79.2	68.1	8.72	9.76
<i>L. deliciosus</i>	140.8 ± 11.0	17.8 ± 0.1	561.2 ± 26.5	56.1	9.99	4.09
<i>L. edodes</i>	290.43 ± 20.6	15.4 ± 0.4	563.7 ± 33.6	56.3	8.68	20.32
<i>M. conica</i>	143.4 ± 3.5	16.5 ± 0.9	552.4 ± 53.1	55.2	9.11	5.23
<i>P. eryngii</i>	301.2 ± 16.0	16.8 ± 0.2	593.8 ± 30.1	59.3	9.98	20.14
<i>P. pulmonarius</i>	166.6 ± 3.1	15.4 ± 0.3	585 ± 10.6	58.5	9.01	7.65

For certain fungal species, MAE seems to be a more efficient method to obtain polysaccharides than conventional methods or other advanced techniques but not for all. Subcritical water extractions (SWE) extracted higher polysaccharide amounts than MAE from *P. ostreatus* but not significant differences were noticed when the polysaccharides were extracted from *G. lucidum* [12]. Moreover, *L. edodes* showed a 21% PSCs extraction yield using PWE at higher temperatures (200 °C and 10.7 MPa) but 4.71% was recovered when extraction was carried out at 150 °C [24], values very similar to MAE where a 19.1% yield was noticed. Ultrasound-assisted extractions (UAE) were also tested using several mushrooms, however, they were usually less effective than MAE, for instance UAE from the latter mushroom

extracted 9.75% PSCs, meaning 1.6 fold increase compared to conventional hot water extraction but still lower than observed using MAE [31].

Adjustments of the fluorimetric method for fungal (1→3)- β -D-glucans determination

The fluorimetric method is based on the sirofluor preference for binding to (1→3)- β -D-glucans. Although an increased signal emission intensity can be expected with higher number of these branches, there is not a direct correlation since the fluorescence intensity is affected by the polysaccharide structure [17]. Ko & Lin (2014) [18] also indicated the influence of the structure and conformation of (1→3)- β -D-glucans to the fluorescence profiles of nine standards, e.g. pachymaran, yeast glucans, curdlan. Therefore, the maintenance of the β -D-glucan native structure during their extraction process is critical for a reliable identification and quantification. However, in previous studies, insufficient attention was given to sample preparation to keep the integrity of these molecules, usually a conventional protocol was followed [18]. Thus, the fluorimetric method used to determine fungal (1→3)- β -D-glucans was adapted at several stages, from the sample preparation to the selection of the adequate standard compound for the β -D-glucan quantification.

The PEFs obtained with MAE were not completely soluble in water at room temperature but in alkalis. However, a careful post-extraction treatment of the samples should be carried out, avoiding drastic changes that could compromise the chemical integrity of the β -D-glucans. Treatment with sodium hydroxide improves β -D-glucans separation from impurities and facilitates their quantification but it could modify their helical 3D conformations [4, 32] and in high concentrations could damage even the primary structure. Therefore, a solution of 0.05 M instead 1 M NaOH [18] was used to dissolve β -D-glucans since the lower concentration was able to dissolve the samples and standards. NaBH₄ (1% w/v) was also added to the NaOH solution to protect the polysaccharide chains from degradation [33]. Indeed, addition of NaBH₄ enhanced 17% the fluorescence intensity of curdlan compared to a similar solution without the reducing agent. Moreover, previous studies remarked the importance of the incubation step at 80 °C for 30 min to enhance the complex

between sirofluor and the polysaccharides with (1→3)- β -D-glucopyranose-linkages [18]. However, if lower temperatures were tested (down to 50°C), no differences in fluorescence intensity were noticed, therefore, fifty degrees were selected to protect the polysaccharides from thermal de-polymerization.

Isolated polysaccharides including the β -D-glucans-linkages frequently described in mushrooms were selected as representative standards to adjust the fluorimetric method. When these β -D-glucans were treated according to the modified method, linear (1→3)- β -D-glucans such as curdlan emitted intense fluorescence, but moderate fluorescence was noticed for branched (1→3),(1→6)- β -D-glucans (Figure 3a). Schizophyllan and PUL were both (1→3)- β -D-glucans with (1→6)- β -D-Glc unit branching respectively every 3 and 2.7 residues of the backbone chain [21, 34] and therefore, they showed slight differences in their degree of branching (DB) (0.33 for schizophyllan and 0.37 for PUL). Moreover, when β -D-glucans from *Saccharomyces cerevisiae* were analyzed (DB = 0.03-0.2) [35], an intermediate fluorescence was noticed between curdlan (DB = 0) and schizophyllan suggesting that higher DB might fold the molecules more compact or tighter, impairing the binding of the fluorophore to the (1→3)-linkages of the backbone chain. This effect might result in a fluorescence reduction and indeed, PUL showed slightly lower fluorescence intensity than schizophyllan. Computer modeling studies using glucans with different configurations (α or β) and linkages, suggested that (1→6)- β -D-glucans might show highly flexible 3D conformation due to an easy rotation freedom between glucose residues, while (1→3)- β -D-glucans showed helical and flexible conformations (including also glucans with O-6-branches) [36]. These observations indicated that the helical conformation was essential for the sirofluor fluorescence stimulation. Thus, a linear (1→6)- β -D-glucan (B6G) and a mannogalactan (MG) were also tested under the adjusted conditions and both lacked fluorescence. This result confirmed that still after the protocol modifications, sirofluor was binding to β -D-glucans with helical conformations including linear or/and branched (1→3)- β -D-glucans.

Since each mushroom species synthesizes its own particular set of β -D-glucans, a wide variety of primary structures, degrees of branching and conformations are described [35]. When the PEF obtained by MAE from several selected species

were analyzed to detect the fluorescence of their β -D-glucans, a range of responses was noticed (Figure 3b), leading to slightly similar slope values. The lowest and highest data were respectively for *M. conica* and *A. caesaria* (Table 3) showing fluorescence values closer to PUL than to schizophyllan or curdlan. Obviously, the extracts contained many β -D-glucans showing several structures and conformations that were all contributing to the fluorescence intensity while signals from curdlan and schizophyllan are exclusive.

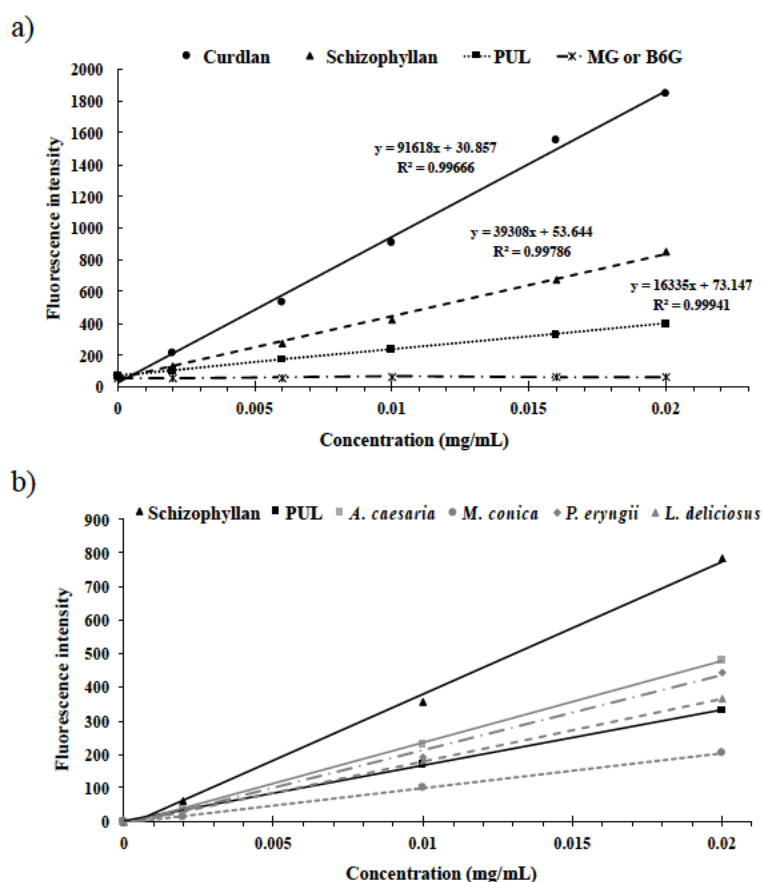


Figure 3. Fluorescence intensity of a) standard β -D-glucans and a mannogalactan (MG) b) the PEFs extracted by MAE from several mushroom species. The regression equation for the standards are included.

However, only curdlan (linear (1→3)- β -D-glucan) is frequently used as standard for the β -D-glucan quantification regardless the mushroom source and most of the edible species (basidiomycetes) contain larger amounts of branched (1→3),(1→6)- β -D-glucans than (1→3)- β -D-glucan (more common in ascomycetes) [2]. Thus, by using curdlan as standard, the β -D-glucan concentration of many mushroom species might be underestimated or misunderstood. For instance, the β -D-glucan content for *A. aegerita* PEF were 140.1 mg/g using curdlan and 522.3 mg/g using a (1→3),(1→6)- β -D-glucan (PUL) as standards (Table 3). For most of the tested species, results obtained using curdlan or PUL were significantly different (one-way ANOVA analysis ($p < 0.05$)) (Table 3). Thus, since the major compounds in basidiomycetes are branched (1→3),(1→6)- β -D-glucans, it should be expected a higher contribution of these structures to the total polysaccharide fractions than linear glucans. Therefore, PUL might be a more suitable compound to be used as standard than curdlan, even schizophyllan could also be adequate and more convenient since it is commercially available and would induce a lower quantification error than curdlan (Figure 3b).

For the same reason, depending on whether PUL or curdlan are used as standard (Table 3), the PEFs composition might be completely different. For instance, the (1→3)- β -D-glucans represented 14.8% of the polysaccharides from *A. judae* PEF when curdlan is used as standard while using PUL, 63.2% of the PEF polysaccharides were (1→3)- β -D-glucans. Similarly, the β -D-glucan percentages found in PEFs seemed to be highly underestimated in other species such as *A. aegerita*, *H. marmoreus*, *M. conica*, etc. These results could be supported by those previously reported by Synytsya & Novak (2013) [35] and Smiderle et al. (2011, 2017) [12, 37] where β -D-glucans were found in higher concentrations than other polysaccharides such as α -glucans, chitins, heteropolysaccharides, etc. If β -D-glucans are quantified using curdlan, their contribution to the total polysaccharide values is quite low to be the predominant compound within the polysaccharide fractions.

However, quantification using PUL could also overestimate their contribution to the polysaccharides content because in the case of species such as *A. caesarea*, *P. eryngii* and *B. edulis* the estimation of β -D-glucans was almost 2-fold the

amount of total polysaccharides. Nevertheless, the phenol-sulfuric acid method estimates the PSC concentration mainly because glucose residues are generated with the digestion, if other monosaccharides (xylose, mannose, galactose, etc.) are generated, the method precision decreases. These results bring a suspicious about the suitability of the fluorimetric method for the quantification of β -D-glucans in a complex polysaccharide matrix.

Table 3. Fluorescence (emission at 503 nm) of PEFs extracted by MAE from several edible mushrooms and their (1 \rightarrow 3)- β -D-glucans concentration depending on the standard utilized (curdlan or PUL). n=2 MAE extracts per species; n=2 fluorimetric measurements per PEF; n.d. no signal detected. The linear fitting of the curves (R^2) was 0.99 for all the mushroom species. *Data statistically different (one-way ANOVA, $P > 0.05$, 95% confidence level) between standards for the same species.

Specie	Fluorescence results (AU)				(1 \rightarrow 3)- β -D-glucans (mg/g) in PEFs		(1 \rightarrow 3)- β -D-glucans of PSC (%)	
	0.002 mg/mL	0.01 mg/mL	0.02 mg/mL	Curve slope	Curdlan	PUL	Curdlan	PUL
<i>A. judae</i>	30.25 \pm 11.5	159.5 \pm 56.6	295 \pm 86.3	14829	142.7 \pm 49.3	610.0 \pm 234.1	14.8	63.2
<i>A. aegerita</i>	18.7 \pm 6.6	135.7 \pm 11.1	298.2 \pm 15.3	14637	140.15 \pm 8.62*	522.6 \pm 37.0*	20.5	76.5
<i>A. caesaria</i>	37 \pm 7.7	231 \pm 43.3	481.5 \pm 78.0	24266	245.94 \pm 43.1*	1249.9 \pm 241.6*	38.6	196.4
<i>B. edulis</i>	37.7 \pm 10.6	207.5 \pm 55.0	446.2 \pm 110.6	22401	229.21 \pm 63.2	1020.3 \pm 300.1	38.9	173.3
<i>C. cibarius</i>	n.d.	n.d.	n.d.		n.d.	n.d.	-	-
<i>C. cornucopioides</i>	n.d.	n.d.	n.d.		n.d.	n.d.	-	-
<i>C. lutescens</i>	n.d.	n.d.	n.d.		2.12 \pm 2.12	n.d.	0.3	-
<i>C. tubaeformis</i>	n.d.	n.d.	n.d.		n.d.	n.d.	-	-
<i>H. marmoreus</i>	17.2 \pm 4.3	115 \pm 10.0	250.7 \pm 33.0	12643	120.0 \pm 18.5*	543.6 \pm 13.8*	17.6	79.8
<i>L. deliciosus</i>	22.7 \pm 5.3	178 \pm 19.6	368.5 \pm 22.1	18716	184.7 \pm 12.4*	713.7 \pm 53.5*	32.9	127.2
<i>L. edodes</i>	23.5 \pm 12.0	135 \pm 0	311.5 \pm 10.6	15593	152.7 \pm 5.9*	647.1 \pm 28.7*	27.1	114.8
<i>M. conica</i>	15.2 \pm 5.7	102 \pm 23.6	204 \pm 42.3	10325	94.5 \pm 23.1	400.5 \pm 129.5	17.1	72.5
<i>P. eryngii</i>	32 \pm 4.9	192.7 \pm 18.0	445 \pm 37.1	22349	226.4 \pm 20.3*	1140.5 \pm 113.8*	38.1	192.1
<i>P. pulmonarius</i>	18.7 \pm 4.8	156.7 \pm 14.4	337.7 \pm 36.2	17153	167.2 \pm 20.3*	639.4 \pm 87.5*	28.6	109.3

On the other hand, the fluorimetric determinations (independently of the utilized standard) indicated that the polysaccharides detected in PEFs obtained from mushrooms belonging to the Cantharellales order (*Cantharellus lutescens*, *Cantharellus cibarius*, *C. cornucopioides* and *C. tubaeformis*) (Table 2) might be different than (1 \rightarrow 3)- β -D-glucans, or contain also other polysaccharides interfering in the sirofluor complexation, as no fluorescence was detected (Table 3).

Chemical characterization of the PEFs extracted by MAE

In order to study the real β -D-glucans contribution to the fluorescence intensity noticed (and to the total polysaccharide values of the PEFs) as well as the method quantification accuracy, the monosaccharide composition and structure of PEFs were monitored.

Table 4. Monosaccharide composition (%) of PEFs obtained by MAE. ^a % of peak area relative to total peak areas, determined by GC–MS; ^b Trace amounts $\leq 0.5\%$.

Species	Monosaccharides (%) ^a					
	Fucose	Xylose	Methyl-Hexose	Mannose	Galactose	Glucose
<i>A. judea</i>	-	3.4	-	14.3	-	82.3
<i>A. aegerita</i>	2.3	-	-	3.6	5.3	88.8
<i>A. caesarea</i>	1.8	-	-	5.8	5.2	87.2
<i>B. edulis</i>	Tr. ^b	2.3	Tr. ^b	22.8	10.1	64.2
<i>C. cibarius</i>	Tr. ^b	12.7	-	35.9	-	51.2
<i>C. cornucopioides</i>	Tr. ^b	15.8	-	33.5	5.9	44.5
<i>C. lutescens</i>	Tr. ^b	13.7	-	38.0	5.3	42.7
<i>C. tubaeformis</i>	Tr. ^b	21.8	-	29.1	4.7	44.1
<i>H. marmoreus</i>	Tr. ^b	1.8	-	9.9	5.5	82.7
<i>L. deliciosus</i>	Tr. ^b	3.1	-	12.0	6.5	78.2
<i>L. edodes</i>	1.6	2.1	-	14.3	4.9	77.0
<i>M. conica</i>	-	-	-	10.3	4.0	85.7
<i>P. eryngii</i>	-	-	2.5	10.8	4.9	81.8
<i>P. pulmonarius</i>	Tr. ^b	1.3	2.6	8.9	6.8	79.8

The PEFs that exhibited fluorescence contained more than 64% glucose in their monosaccharide composition (Table 4). Species that did not show fluorescence, belonging to the genus *Cantharellus*, showed lower glucose content (51.4 - 42.9%) but considerable amounts of mannose (29.1 – 38%) and xylose comparing to the other species. The mushroom from the Boletales order (*B. edulis*) showed higher galactose levels than the rest of selected species. Fucose monosaccharide was only found in few species at levels below 3%. Moreover, methyl-hexose was detected in Pleurotaceae

mushrooms (*P. eryngii* and *P. pulmonarius*) and in *B. edulis* in low amounts. Therefore, the slight differences observed in the monosaccharide composition are not consistent enough to explain the different slopes noticed in the fluorescence regression equations. For example, *P.eryngii* and *H. marmoreus* showed similar monosaccharide composition (~82% glucose, ~5% galactose and ~10% mannose), however, the regression slope of the first one was 1.8 fold the other, suggesting that sugar moieties might not be highly involved in the fluorescence observed (Tables 3 and 4). Wise to take into consideration was the fact that those mushrooms showing high β -D-glucan contents (even higher than their PSC values) i.e. *A. caesarea* or *P. eryngii* also showed high glucose contents (above 80%). The PSC levels of the Chantarellaceae family might also be underestimated because they contained large amounts of other monosaccharides that are not glucoses (e.g. *C. lutescens* contains 38% mannose and 13.7 % xylose) adding larger errors to the colorimetric determination.

Signals observed in the HSQC spectra from PEFs were in concordance with the monosaccharides determined for each species. The most representative spectra are compiled in Figure 4, while the others are supplied as supplementary material (Supplementary Figure 3). With exception of *M. conica*, spectra from *A. caesarea*, *C. lutescens*, *L. edodes* (Figure 4a, b and c), *A. aegerita* or *B. edulis* (Supplementary Figure 3), showed intense signals at δ 102.8/4.52 and 102.8/4.72 relative to C-1 of β -D-Glcp; at δ 85.0/3.75 relative to C-3 *O*-substituted and; at δ 69.0/4.19 and 69.0/3.86 relative to CH₂ *O*-substituted of the same units. These data suggest the presence of β -D-glucans (1 \rightarrow 3)-(1 \rightarrow 6)-linked [38]. The O-6 substitution was confirmed by inversion of CH₂ signals of DEPT-HSQC experiment (data not shown). Linear (1 \rightarrow 3)- β -D-glucans signals could be overlapped to the branched β -D-glucans, therefore a separation process might be required to quantify each of them. Besides, signals at δ 99.8/5.36 and δ 77.7/3.64 were intense in *L. edodes* (Figure 4c) and *M. conica* (Figure 4d) spectra, characteristic of C-1 of α -D-Glcp and C-4 *O*-substituted, indicating the presence of glycogen (α -1,4-1,6-D-glucan), the energy stock of fungi [35]. Small intensity signals of α -D-Galp were observed in all spectra at δ 98.0/4.98 and signals of β -D-Manp were observed mainly in *C. lutescens*, *B. edulis*, and *L.*

edodes at $\delta \sim 101.6/5.14$ - $102.3/5.11$. Species from genus *Cantharellus* showed the higher xylose content, that is not commonly observed in mushrooms and its presence was confirmed by signals at $\delta \sim 103.6/4.42$ in the spectra of all *Cantharellus* species (Figure 4b and Supplementary Figure 3).

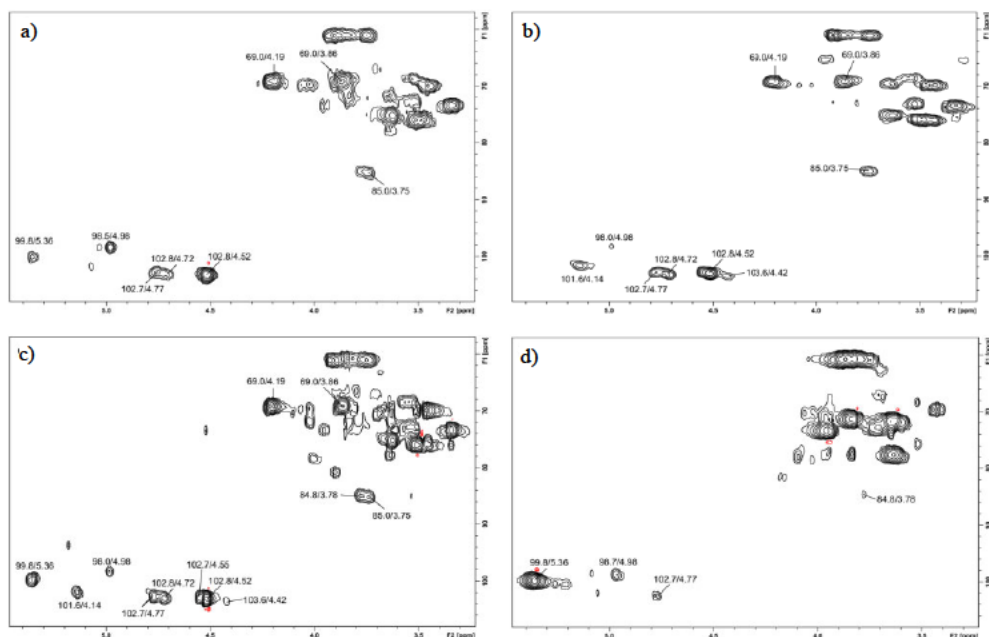


Figure 4. HSQC NMR spectra of a) *Amanita caesarea*, b) *Cantharellus lutescens*, c) *Lentinula edodes* and d) *Morchella conica*. Experiments were performed in D₂O at 70 °C (chemical shifts are expressed in δ ppm).

Mannogalactans, fucomannogalactans and xylomannans were already isolated from other basidiomycetes such as *P. pulmonarius*, *Amanita muscaria*, and *Flammulina velutipes* [21, 39, 40], which confirms the findings in this study. Considering that β -D-glucans are able to complex with sirofluor and *Cantharellus* species did not show fluorescence, it is possible that polysaccharides containing xylose and mannose (present in high amounts in these species) may influence the β -D-glucans 3D conformation present in *Cantharellus* PEFs. These types of polysaccharides were also previously pointed as indirect scavengers of smaller molecules because of their gelling properties so, they could partially attach to sirofluor provoking a lower fluorescence emission [41]. Furthermore, *M. conica*

showed low fluorescence and its HSQC spectrum presented more intense glycogen signals than signals of β -D-glucans in comparison to the other species, indicating that glycogen might also interfere the proper binding of the fluorochrome to the β -D-glucans structures.

Hence, the fluorimetric method seemed to be sensitive enough for the detection of small amounts of (1 \rightarrow 3)- β -D-glucans in polysaccharide mixtures such as MAE extracts, with limits of detection below 2 μ g/mL. So, it might be used as a fast and easy alternative to determine the presence/absence of (1 \rightarrow 3)- β -D-glucans in complex mixtures, being tentatively applicable to other biological sources. However, to be sure that these linkages are not present in the analyzed samples when lack of fluorescence is observed, more exhaustive qualitative techniques (i.e. NMR) must be performed. No specific correlation was obtained between the fluorescence intensity noticed in the PEFs and their chemical composition suggesting that the fluorescence test should not be recommended for an accurate and trustable quantification of the (1 \rightarrow 3)- β -D-glucans content for any mushroom species. The presence of α -glucans and other polysaccharides, their particular interactions and possible complexing between them or the sirofluor, might under/overestimate their real concentrations depending on the species and standard considered.

Conclusions

Microwave-assisted extraction is a friendly environmental technology that could be used to obtain polysaccharide-enriched fractions from edible mushrooms. The most suitable extraction conditions seemed to be applicable to many different species obtaining interesting β -D-glucan yields. Although the fluorimetric method resulted appropriate for β -D-glucan detection, this technique must not be used for quantification analysis and a few details should be taken into consideration when carrying it out. For instance, modifications such as reduction of the alkali concentration utilized for experiments, decrease of the incubation temperature and addition of NaBH₄ improved the polysaccharide stability. Utilization of branched (1 \rightarrow 3),(1 \rightarrow 6)- β -D-glucans (particularly PUL or the commercially available schizophyllan) instead of linear (1 \rightarrow 3)- β -D-glucans such as curdlan lowered the

experimental error because the fluorescence intensity was influenced by the degree of branching (DB) of the β -D-glucans and most of the mushrooms showed (1 \rightarrow 3),(1 \rightarrow 6)-branched structures in high levels. Moreover, in the case that no fluorescence is observed using the aniline-blue method for mushroom extracts a more exhaustive analysis (i.e. NMR) must be carried out before discarding the sample to avoid misinterpretations (as noticed in the *Cantharellus* genus). Perhaps the presence of other polysaccharides and/or their particular tridimensional configurations negatively affected the preference of fluorophore to bind (1 \rightarrow 3),(1 \rightarrow 6)- β -D-glucans. Deeper studies on the tridimensional folding possibilities of (1 \rightarrow 3),(1 \rightarrow 6)- β -D-glucans and their complexing with other polysaccharides are required to clarify this artefact before using the method for these mushroom species.

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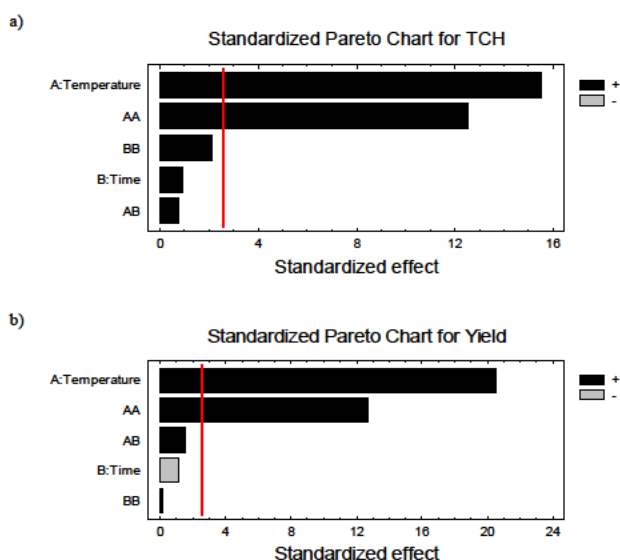
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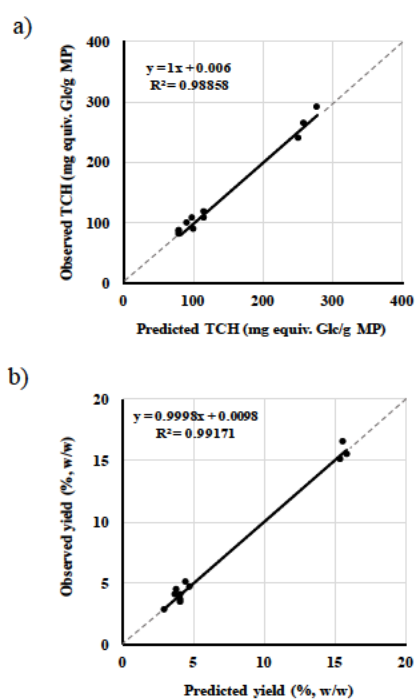
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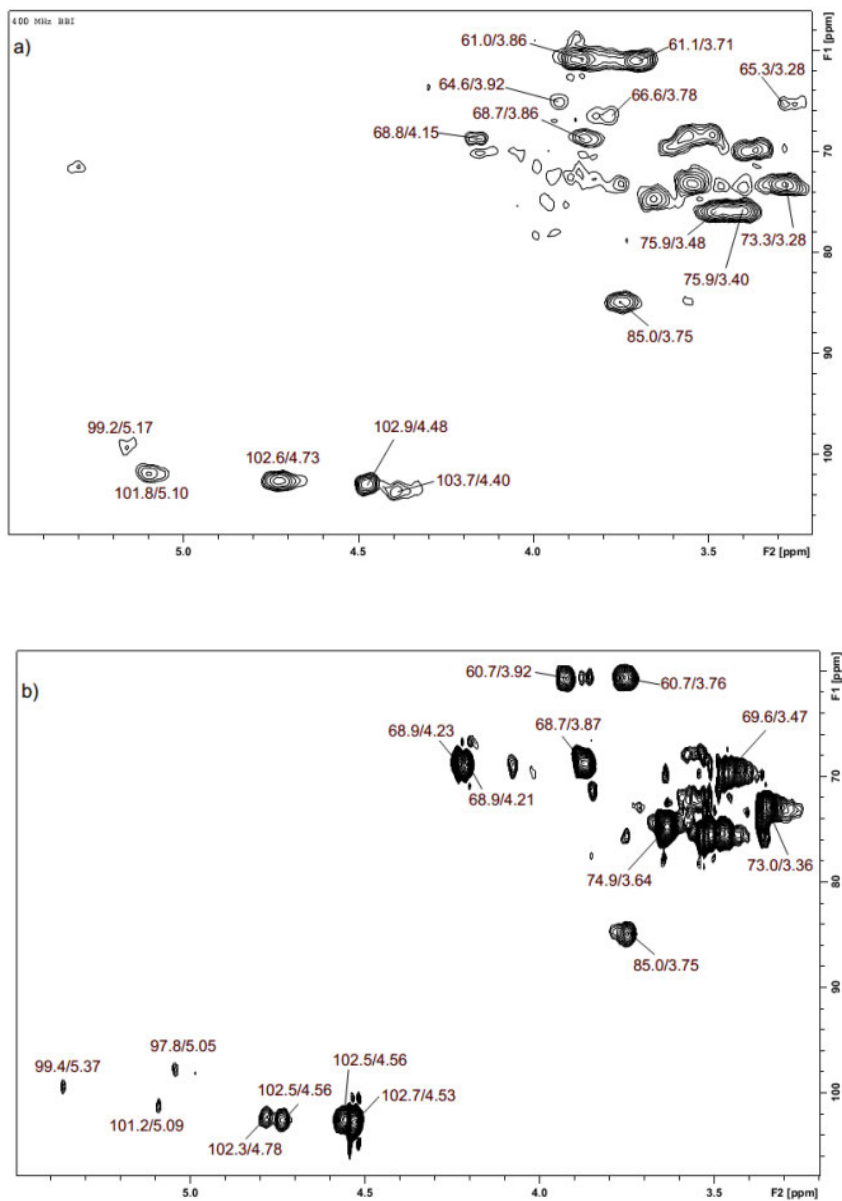
Supplementary information



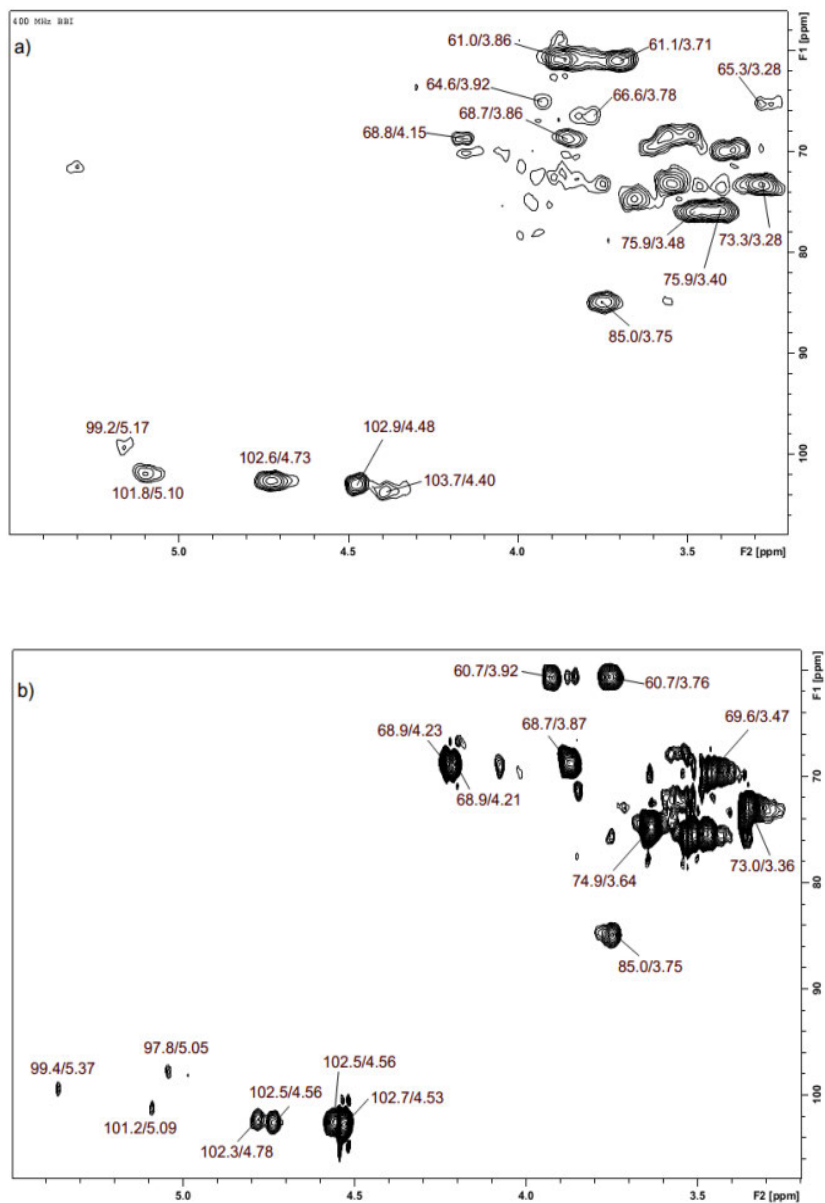
Supplementary Figure 1. Predicted effect of factors *temperature* and *time* and their interactions on a) TCH content and b) the PEF yield, sorted in decreasing order of importance. Red line represents $p=0.05$.



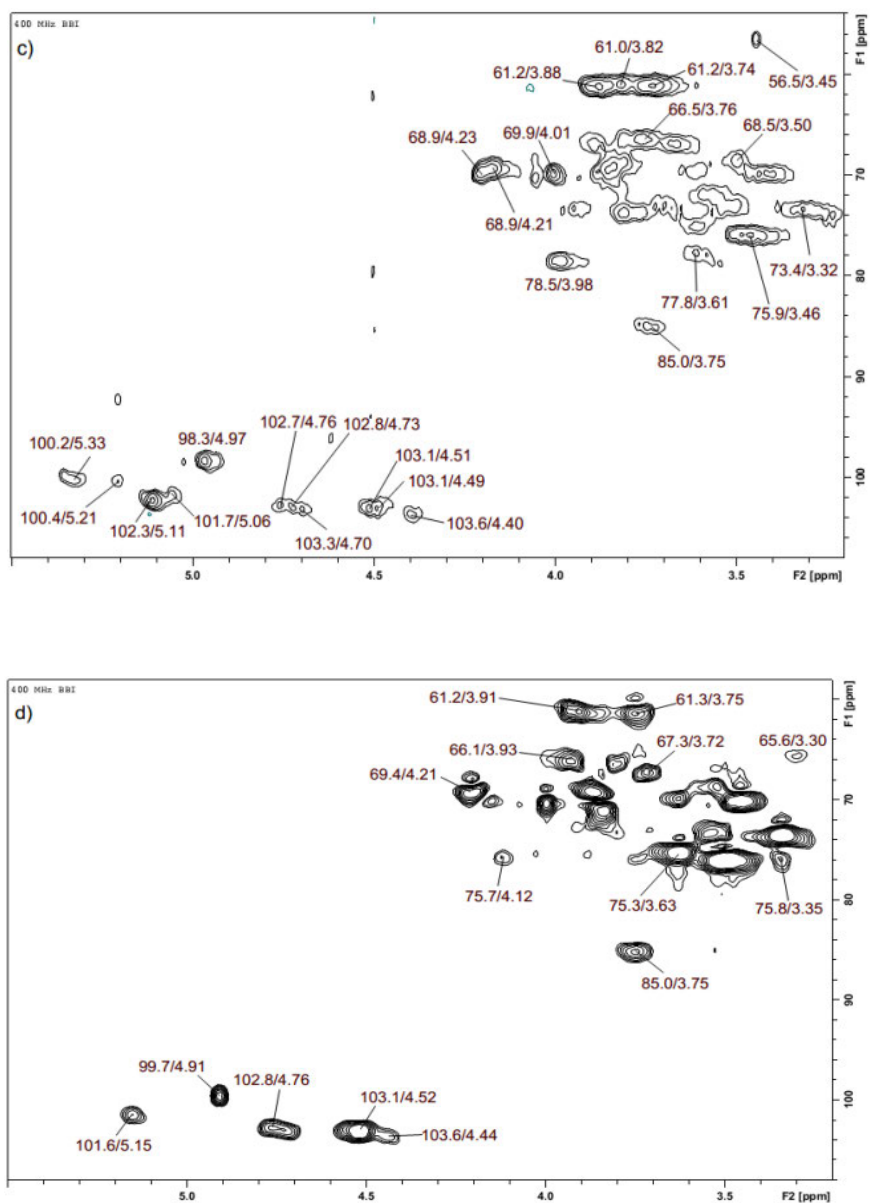
Supplementary Figure 2. Observed vs. predicted values of a) TCH content and b) PEFs yield from *L. edodes* after MAE. Dashed lines correspond to the perfect fit line (y-intercept=0, slope=1).



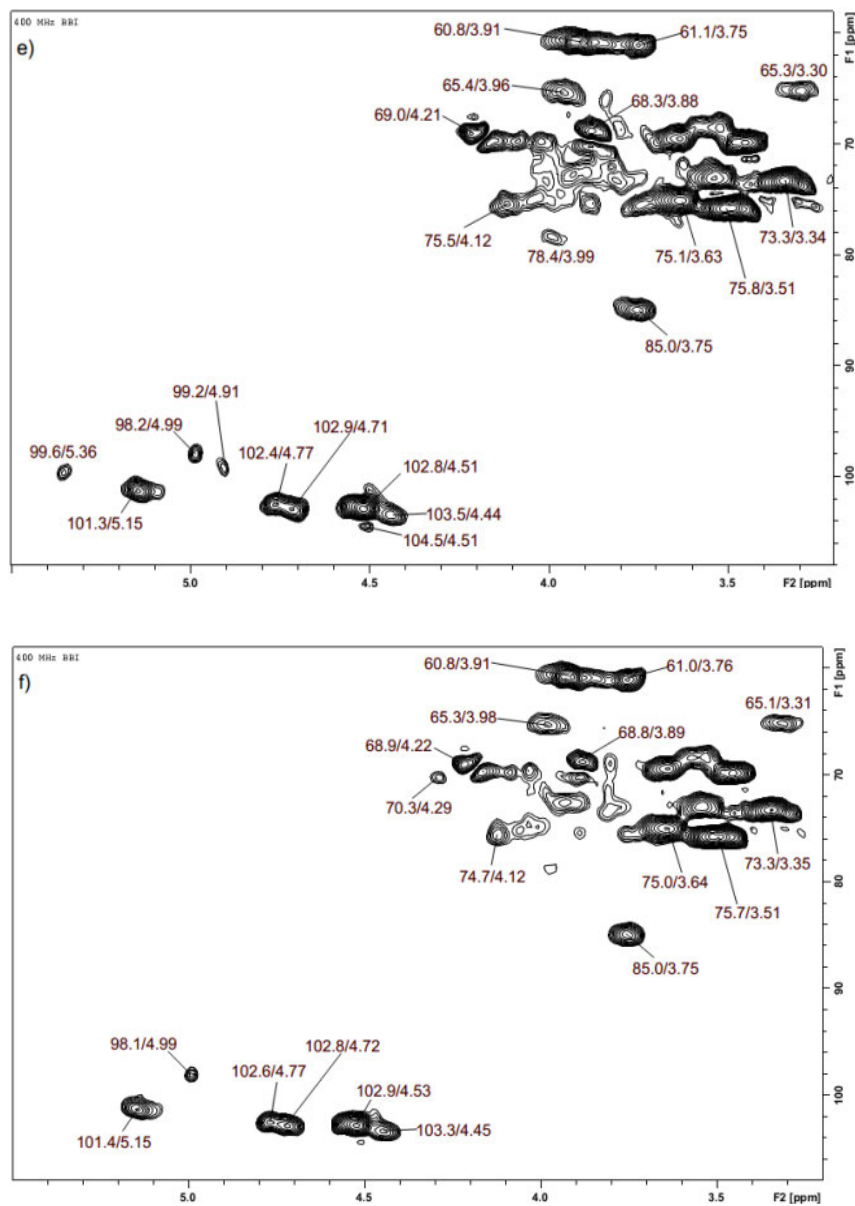
Supplementary Figure 3. (Continued in next page)



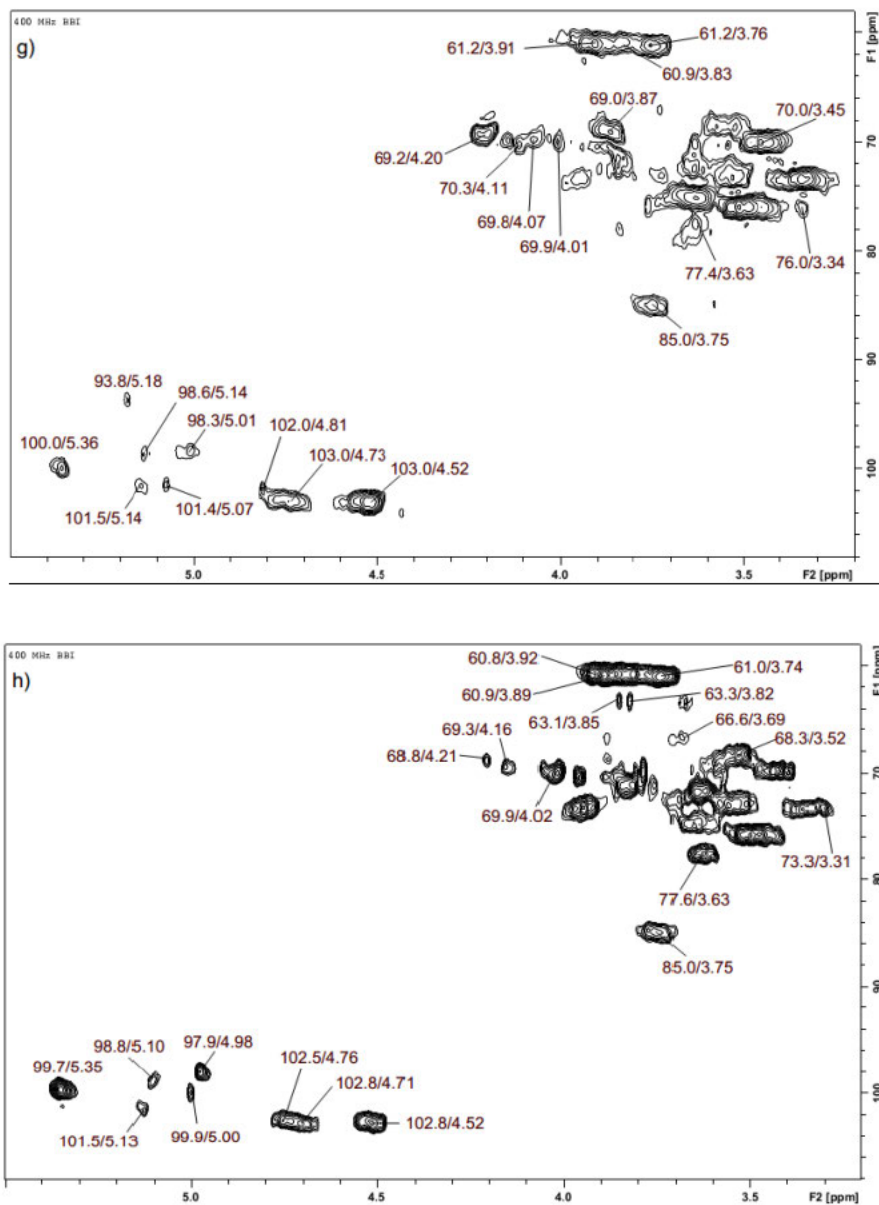
Supplementary Figure 3. (Continued in next page)



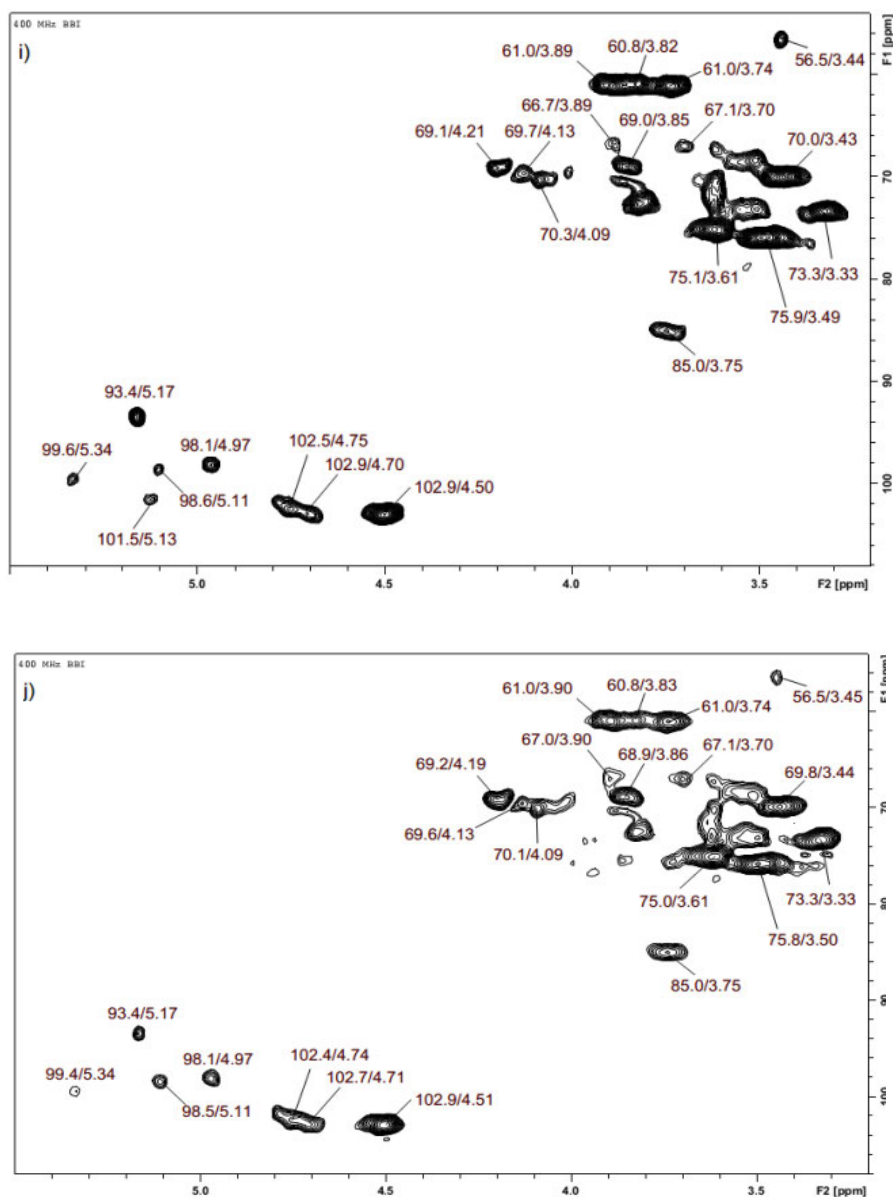
Supplementary Figure 3. (Continued in next page)



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Supplementary Figure 3. HSQC NMR spectra of a) *Auricularia judea*, b) *Agrocybe aegerita*, c) *Boletus edulis*, d) *Cantharellus cibarius*, e) *Cantharellus cornucopioides*, f) *Cantharellus tubaeformis*, g) *Hypsizygus marmoreus*, h) *Lactarius deliciosus*, i) *Pleurotus eryngii* and j) *Pleurotus pulmonarius*. Experiments were performed in D₂O at 70 °C (chemical shifts are expressed in δ ppm).

Manuscript 2

Testing the effect of combining innovative extraction technologies on the biological activities of obtained β -D-glucan-enriched fractions from *Lentinula edodes*

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Abstract

Innovative technologies as ultrasound-assisted extraction (UAE) (550W, 60% amplitude, 50 °C) or subcritical water extraction (SWE) (200 °C, 11.7 MPa) were more effective than hot water extractions to obtain β -D-glucan-enriched fractions from shiitake mushrooms. UAE required longer extraction time (60 min) than SWE (15 min). Combination of UAE+SWE or pre-treatment of the raw material with supercritical CO₂ (SFE) (40 °C, 35 MPa, 3 h) before both extractions yielded extracts containing larger β -D-glucan concentrations. Fluorimetric/colorimetric determinations indicated that obtained fractions contained (1 \rightarrow 3)- and (1 \rightarrow 3),(1 \rightarrow 6)- β -D-glucans. NMR confirmed their presence as well as (1 \rightarrow 3)- α -D-glucans and heteropolymers including mannose and galactose. SWE (15 min), SFE+SWE or UAE+SWE extracts showed larger glucose levels and lower mannose and galactose residues than the other extractions suggesting certain extraction specificity towards β -D-glucans. They also included more chitin derivatives than UAE. The extracts obtained after combination of technologies partially retained their immunomodulatory properties but they showed high hypocholesterolemic activities according to *in vitro* studies.

Introduction

Lentinula edodes is an Asian edible mushroom consumed worldwide and commonly known as shiitake mushroom. Traditionally, it was included in popular remedies to prevent diseases or to promote health and wellbeing and, in the last decades, scientific studies confirmed that indeed, the mushroom contains many bioactive compounds that might reduce the risk of harmful disorders such as cancer, cardio- or cerebrovascular diseases, etc. [1]. Fungal β -D-glucans are compounds thoroughly investigated because they are considered as dietary fiber (together with chitins) with anti-inflammatory, immunomodulatory, antioxidant, anti-tumoral, hypocholesterolemic, antimicrobial activities, etc. [1-6] and therefore, they might be used to design novel functional foods.

Mushroom β -D-glucans are structurally different than those from plants or bacteria since they have a main chain of β -D-glucose units (1 \rightarrow 3)-linked with different branching usually at O-6 position by β -D-glucose units or other oligosaccharides. Their branching degree determines the tertiary structure of the glucan: (1 \rightarrow 3)- β -D-glucans with few or no branches mainly present a single linear structure while highly branched (1 \rightarrow 3),(1 \rightarrow 6)- β -D-glucans show a triple helix conformation [7]. In shiitake mushrooms, lentinan is the major (1 \rightarrow 3),(1 \rightarrow 6)- β -D-glucan and showed many beneficial properties for human health [8, 9]. Since the integrity of the molecules is important to keep many of their beneficial properties, the extraction methods utilized to generate β -D-glucan-enriched extracts should be carefully selected because they might modify their molecular weight or tridimensional structure [10].

Although some fungal polysaccharides are polar and easily extractable with water, many β -D-glucans require aggressive treatments such as hot water or alkaline solutions and others remain in the insoluble residue (Chapter 3, Manuscript 3) [11]. Moreover, chitins are water insoluble polymers and only low molecular weight derivatives or degradation products could be extracted using conventional hot water procedures (Chapter 3, Manuscript 3; Chapter 4, Manuscript 1) [11]. To obtain chitinous materials, more drastic treatments are required to induce dissolving of other components in alkaline or acid solutions and the precipitation of chitin-enriched

fractions [12, 13]. Novel advanced technologies such as ultrasound-assisted extractions (UAE) or subcritical water extraction (SWE) facilitate the extraction of these and other polysaccharides using water as non-pollutant solvent [14, 15]. Ultrasound-assisted extractions generate ultrasonic waves provoking implosions of the produced cavitation bubbles, causing disruption of fungal cell walls. This treatment enhanced mass transfer and extraction yields [16, 17] and it was successfully used to extract polysaccharides from several mushrooms (*Ganoderma lucidum*, *Agaricus bisporus*, *Boletus edulis*, etc.) and their by-products [18, 19]. Pressurized liquid extractions constitute other interesting approach to enhance polysaccharide extraction, particularly SWE. When subcritical pressures are used, the solvent is heated above its boiling point keeping its liquid state and conferring it different properties than those showed at room temperature and atmospheric pressure. SWE modify dielectric constant of water and decrease its viscosity being able to solubilize non-polar compounds such as large size polysaccharides (when temperature is above 100 °C) [20, 21, 22]. Mushrooms polysaccharide-enriched extracts were obtained by SWE from many species such as *Agaricus bisporus*, *Pleurotus ostreatus*, *Ganoderma lucidum*, etc. [22-24].

Besides the previously mentioned, other green technologies were used to extract interesting compounds from edible mushrooms. Supercritical fluid extractions (SFE), particularly those using supercritical CO₂ as solvent, were mainly applied to selectively extract lipids from food matrices because of the low polarity of the solvent [25]. Therefore, it might also be used to remove fat components from fungal extracts enhancing, in a subsequent step, polysaccharide extraction, as described in previous works for *Antrodia camphorata* and *Agaricus blazei* mycelia [26, 27]. Combinations of different extraction technologies to obtain β -D-glucan-enriched extracts were also tested and higher yields than single extractions were usually noticed. For instance, in a complex extraction reactor, the use of pressurized hot water combined with sub/supercritical CO₂ increased 1.26 folds β -D-glucan yields obtained from *Ganoderma lucidum* than using only pressurized hot water [28]. Moreover, ultrasonic/microwave assisted extractions (UMAE) were also more effective than hot water extractions to obtain bioactive polysaccharides from *Inonotus obliquus* [29] and combined with enzymes facilitated the extraction even more [30].

Therefore, in this work, UAE and SWE were firstly carried out at different extraction times to define the most adequate to obtain β -D-glucan-enriched extracts from shiitake mushrooms. The extraction yields were compared with more conventional extractions. Afterwards, combinations of UAE and SWE as well as pre-treatments with SFE were investigated as innovative protocols to improve β -D-glucan extraction yields. The nature of bioactive polysaccharides from the extracts was determined by different methodologies (enzymatic, fluorimetric and colorimetric methods, HPSEC, GC-MS, NMR) and their biological activities studied *in vitro* (hypocholesterolemic and immunomodulatory activities) to investigate whether the extraction procedure modify their beneficial properties.

Materials and methods

Biological material

Powdered *Lentinula edodes* S. (Berkeley) fruiting bodies (particle size < 0.5 mm, moisture < 5%) were purchased from Glucanfeed S.L. (La Rioja, Spain) and stored in darkness at -20 °C until further use.

Reagents

Absolute ethanol and sulfuric acid (H_2SO_4) were obtained from Panreac and phenol, sodium borohydride (NaBH_4), sodium hydroxide pellets, glycine, D-glucose, glucosamine hydrochloride, aniline blue diammonium salt 95%, hydrochloric acid 37%, acetylacetone, p-dimethylaminobenzaldehyde, trifluoroacetic acid, pyridine, acetic anhydride, copper(II) sulfate (CuSO_4), deuterated dimethylsulfoxide ($\text{Me}_2\text{SO}-d_6$), Congo Red, citric acid, RPMI 1640 medium and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich Quimica (Madrid, Spain). CO_2 was supplied by Air-Liquid, S.A. (Madrid, Spain), schizophyllan was obtained from Contipro Biotech (Dolni Dobrouc, Czech Republic) and synthetic soluble starch was acquired from Scharlab (Barcelona, Spain).

Supercritical CO₂ extraction pre-treatment (SFE)

Shiitake powder (253 g) was mixed with 1.9 kg of 6 mm diameter stainless steel spheres (ratio 1:1 (v/v) powder:spheres) in a 2 L extraction cell connected to a supercritical fluid extraction plant (model SF2000, TharTechnology, Pittsburgh, PA). Pressurized CO₂ was applied into the loaded cell at 35 MPa and 40 °C with a 3.6 kg/h recirculating flow during a total extraction time of 3 h as they were the conditions described as adequate for fungal lipids (Chapter 1, Manuscript 1). The lipophilic material solubilized in supercritical CO₂ was removed in separators [31]. After those 3 h, the pressurized material remaining in the extraction cell (called SFE fraction) was separated from steel spheres by sieving in a sieve shaker (Cisa BA200 N, Barcelona, Spain) and stored at -20 °C until further use.

Ultrasound-assisted extractions (UAE)

Shiitake powder (1 g) was mixed in MilliQ water (100 mL) and submitted to sonication (Branson SFX550 Digital Sonifier 550W, Branson Ultrasonics, USA) using an ultrasonic probe (1/2'') and selecting 60% amplitude for sonication output. During processing, the temperature was maintained at 50 °C using a water bath with ice and a thermometer and samples were taken after 15, 30, 45 and 60 min extraction in duplicate. The obtained mixtures were submitted to vacuum filtration (through Whatman filter paper no. 1) to separate the soluble fraction from the insoluble residue. Afterwards, soluble fractions were lyophilized (UAE fractions) using a freeze-dryer LyoBeta 15 (Telstar, Madrid, Spain) and stored at -20 °C until they were further processed to obtain polysaccharide-enriched extracts or submitted to subcritical water extractions.

The UAE fractions were mixed with water (50 g/L) and three volumes of ethanol to induce polysaccharide precipitation and left incubating overnight at 4 °C. The precipitates were collected by centrifugation (10 000 rpm, 10 min, 4 °C) in a Thermo Scientific Heraeus Multifuge (Thermo Fisher Scientific, Madrid, Spain) and freeze-dried. The obtained polysaccharide-enriched extracts (UAE extracts) were stored at -20 °C until further analysis.

Subcritical water extractions (SWE)

Shiitake powder (0.5 g) was mixed with washed sea sand (Panreac, Barcelona, Spain) at ratio 1:8 (mushroom:sand, w/w) and placed in an extraction cell (11 mL) covered with cellulose filters (Dionex Corporation, USA) from an Accelerated Solvent Extractor (ASE) 350 (Dionex Corporation, USA). Extractions were carried out in duplicate with MilliQ water at 200 °C and 11.7 MPa but using different extraction times (15, 30, 45 and 60 min). Temperature was fixed at 200 °C as it was previously pointed as the ideal temperature to extract polysaccharides from shiitake mushrooms [23]. The obtained mixtures were vacuum filtrated and the soluble fractions were freeze-dried (SWE fractions) and used to generate polysaccharide-enriched extracts (SWE extracts) as above described.

Conventional extractions

Two different conventional extraction methods were followed to compare with the previously mentioned advanced technologies. One was a simple hot water extraction (HWE) and the other was a method frequently used to obtain polysaccharides using an autoclave (steam pressurized extraction, SPE).

Shiitake powder (0.5 g) was mixed in MilliQ water (50 mL), placed into a water bath at 100 °C and vigorously stirred during 15, 30, 45 and 60 min. Incubations were carried out in duplicate. Afterwards, the obtained mixtures were vacuum filtrated and the soluble fractions freeze-dried. Obtained HWE fractions were later submitted to polysaccharide extraction as previously described to obtain hot water extracts (HWE extracts).

Similarly, the same shiitake mixture was heated at 120 °C for 20 min in an autoclave and cooled down to 4 °C following the method reported by Jeurink et al. (2008) [32]. After the steam pressurized extraction (SPE), the mixture was also submitted to vacuum filtration and the soluble fraction freeze-dried (SPE fraction) and further processed to obtain SPE extracts as indicated above for HWE extracts.

Combined extractions

Sequential extractions were also tested as indicated in Figure 1 by combining SFE pre-treatment (35 MPa, 40 °C, 3 h) with ultrasound assisted extraction or subcritical water extraction. Thus, obtained SFE fraction was submitted to UAE (550W, 50 °C, 60% amplitude) during 60 min as previously indicated for shiitake powder to generate a new SFE+UAE fraction that was further processed to obtain a polysaccharide-enriched extract (SFE+UAE extract) as also described for UAE extracts. Similarly, SFE fraction was submitted to SWE (200 °C, 11.7 MPa, 15 min) obtaining a SFE+SWE fraction and a SFE+SWE polysaccharide-enriched extract (SFE+SWE extract). Moreover, the UAE fractions (550W, 50 °C, 60% amplitude) obtained after 60 min sonication were also submitted to subcritical water extraction (200 °C, 11.7 MPa) during 15 min to obtain the UAE+SWE fractions. Afterwards, polysaccharide-enriched extracts (UAE+SWE extracts) were also generated following the same procedure as previously described for UAE extracts. All these combined extracts were prepared in duplicate and stored as indicated for the simple extracts.

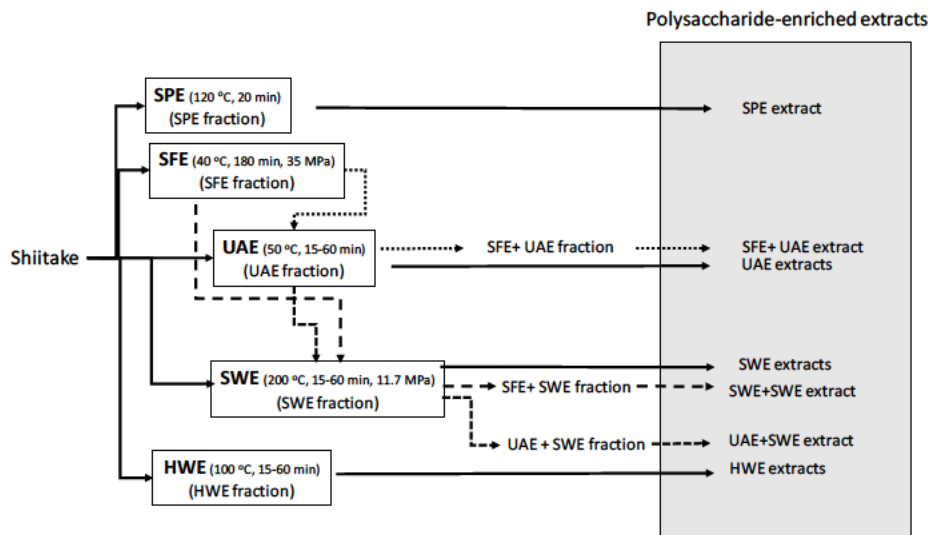


Figure 1. Extraction workflow followed to obtain different fractions and extracts from shiitake powder. Continuous lines indicate single extractions and dotted lines indicate the order followed when two technologies were combined. (SPE, steam pressurized extraction; SFE, supercritical fluid extraction; UAE, ultrasound assisted-extraction; SWE, subcritical water extraction).

Determination of carbohydrates

Total carbohydrate content of shiitake, the extracted fractions and the obtained polysaccharide-enriched extracts was determined by the phenol-sulfuric acid method as described by Fox & Robyt (1991) [33].

Total β -D-glucan concentration was determined in the polysaccharide-enriched extracts using a mushroom and yeast specific β -D-glucan determination kit (β -glucan Assay Kit Megazyme®, Megazyme, Wicklow, Ireland) following the instructions of the user's manual. Samples absorbance was measured using a Genesys 10 UV spectrophotometer (Thermo Fisher Scientific, Madrid, Spain).

The amount of (1 \rightarrow 3)- β -D-glucans in the extracts was determined by the fluorimetric method described by Ko & Lin (2004) [34] but including the modifications that were described in Chapter 3, Manuscript 1 and Smiderle et al. (2017) [22]. The measurements were performed in a M200 Plate Reader (Tecan, Männedorf, Switzerland) with excitation and emission wavelengths set at 398 and 502 nm respectively. The (1 \rightarrow 3),(1 \rightarrow 6)- β -D-glucan content of the extracts was analyzed following the colorimetric method (523 nm) described by Nitschke et al. (2011) [7]. Schizophyllan was used as standard for both determinations.

Chitin content was determined according to Smiderle et al. (2017) [22]. Briefly, precipitated extracts were hydrolyzed with 6 M HCl at 100 °C for 2 h. After the hydrolysis, samples were cooled down and adjusted to pH 10.0. Then, hydrolyzed samples (250 μ L) were treated as described by Rementeria et al. (1991) [35] and absorbance was measured at 530 nm. A glucosamine hydrochloride standard curve was used for quantification.

GC-MS analyses

The monosaccharide composition of the polysaccharide-enriched extracts was determined by hydrolyzing the extracts (1 mg) with 2M trifluoroacetic acid at 100 °C for 8 h followed by evaporation to dryness. The dried samples were dissolved in distilled water (100 μ L) and NaBH₄ (1 mg) was added. Then, solution was kept at room temperature overnight to reduce aldose into alditols [36] and later, the samples

were dried and the NaBH_4 excess was neutralized by adding acetic acid and then removed with methanol (twice) under a compressed air stream. Alditols acetylation was performed in pyridine-acetic anhydride (200 μL ; 1:1 v/v) for 30 min at 100 $^\circ\text{C}$. Pyridine was removed by washing with 5% CuSO_4 solution and the resulting alditol acetates were extracted with chloroform. The samples were injected into an SH-Rtx-5ms (30 m x 0.25 mm ID x 0.25 μm thickness phase). The column was connected to a GC-2010 Plus gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a Combipal autosampler (AOC 5000) and coupled to a triple quadrupole mass spectrometer TQ 8040. The injector and ion source were held at 250 $^\circ\text{C}$ and helium at 1 mL/min was used as carrier gas. The oven temperature was programmed from 100 to 280 $^\circ\text{C}$ at 10 $^\circ\text{C}/\text{min}$ with a total analysis time of 30 min. The samples were prepared in hexane with 1 μL being injected with a split ratio of 1:10. The mass spectrometer was operated in the full-scan mode over a mass range of m/z 50-500 before selective ion monitoring mode, both with electron ionization at 70 eV. Selective ion monitoring mode was used for quantification and *GCMS solution* software (Tokyo, Japan) was used for data analysis. The obtained monosaccharides were identified by their typical retention time compared to commercial available standards. Results were expressed as mol%, calculated according to Pettolino et al. (2012) [37].

HPSEC analyses

Polysaccharide-enriched extracts were injected into a high-performance size-exclusion chromatography system (HPSEC) (Waters, Massachusetts, USA) coupled to refractive index detector (Waters). Four gel-permeation Ultrahydrogel columns in series with exclusion sizes of 7×10^6 , 4×10^5 , 8×10^4 and 5×10^3 Da were used. The eluent was 0.1 aqueous NaNO_2 containing 200 ppm aqueous NaN_3 at 0.6 mL/min. The extracts were dissolved in the eluent (1 mg/mL), filtered through a 0.22 μm membrane and then, injected (100 μL loop). Data were analyzed using Astra software version 4.70.

NMR analyses

NMR spectra (^1H , ^{13}C and HSQC-DEPT) from precipitated extracts were obtained using a 400 MHz Bruker model Advance III spectrometer with a 5 mm inverse probe. The analyses were performed at 70 °C and the samples (30 mg) were dissolved in $\text{Me}_2\text{SO}-d_6$. Chemical shifts are expressed in ppm (δ) relative to $\text{Me}_2\text{SO}-d_6$ at 39.7 (^{13}C) and 2.40 (^1H).

Determination of HMGCR inhibitory activity

The polysaccharide-enriched extracts were solubilized in water (50 mg/mL) and applied (20 μL) into a 96-wells plate. Their inhibitory activity was measured using the commercial HMGCR (3-hydroxy-3-methylglutaryl coenzyme A reductase) activity assay (Sigma-Aldrich, Madrid, Spain) according to the manufacturer's instructions by monitoring their absorbance change (340 nm) at 37 °C using a 96-wells microplate reader BioTek Sinergy HT (BioTek, Winooski, USA). Pravastatin was used as a control for positive inhibition.

Macrophage cultures and immunomodulatory testing

The human monocyte THP-1 cell line was obtained from ATCC and cultured with supplemented RPMI 1640 medium (10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin, 2mM L-glutamine and 0.05 mM β -mercaptoethanol). For differentiation into macrophages, THP-1 cells were seeded (5×10^5 cells/mL) in 24 well-plate with 100 ng/mL phorbol 12-myristate 13-acetate (PMA) and maintained for 48 h at 37 °C under 5% CO_2 in a humidified incubator.

Firstly, the extracts cytotoxicity was evaluated in differentiated macrophages using 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide (MTT) according to Mosmann (1983) [38]. Afterwards, the macrophages were washed with PBS and then replaced with serum-free medium containing LPS (0.05 $\mu\text{g/mL}$) and subtoxic concentrations of the shiitake extracts. After 10 h of incubation, cells supernatants were collected and store at -20°C until use.

Pro-inflammatory cytokines TNF- α (Tumour necrosis factor alpha), IL-1 β (Interleukin 1 beta) and IL-6 (Interleukin 6) were measured in the supernatants by BD Biosciences Human ELISA set (Aalst, Belgium) following the manufacturer's instructions. The quantification was calculated considering positive controls (cells stimulated with LPS) as a 100% cytokine secretion. The colour generated was determined by measuring the OD at 450 nm using a multiscanner autoreader (Sunrise, Tecan). Experiments were carried out in triplicate.

Statistical analyses

Differences were evaluated at 95% confidence level ($P \leq 0.05$) using a one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison test. Statistical analysis was performed using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA).

Results and discussion

UAE and SWE as individual extraction methodologies

Polysaccharide extractions using ultrasounds or subcritical water were carried out at different extraction times and compared with conventional methodologies such as hot water extractions (HWE). When the obtained yields were evaluated (Table 1), results indicated that SWEs extracted more material from shiitake powder than UAEs, almost 2 fold more than HWEs at any extraction time and also more than a commonly utilized method to isolate polysaccharides using water at 120 °C for 20 min (SPE) [32]. These results might suggest that the high temperature of the pressurized water (that might have easily penetrated in the hyphae because it still maintains its liquid status) was more effective to dissolve and extract fungal compounds than other methods using lower temperatures such as 120, 100 or 50 °C. Similarly, the propagation of ultrasonic waves might have also provided a greater water penetration into the fungal hyphae than more conventional methodologies using higher temperatures.

HWE yields were higher than reported in other studies where extractions were carried out for 60 min at 98 °C (Chapter 3, Manuscript 3; Chapter 4, Manuscript

1). However, the value obtained using the SPE method was in the range of previous works [32]. Worth to notice was the decrease observed on SWEs carried out for more than 45 min. It might indicate clogging of the cellulose filter due to the large amount of extracted material. Therefore, if long extraction periods are required, extraction cycles are encouraged since similar studies but using extraction cycles (200 °C, 5 cycles of 5 min) yielded slightly higher values [23].

The polysaccharides amount detected in UAE obtained fractions (Table 1) were in concordance with previous works where 9.75% polysaccharides were recorded from *L. edodes* after 21 min extraction [39]. Similarly, the SWE fractions obtained after 15 min extraction showed a slightly higher polysaccharide content than noticed by Zhang et al. (2019) [40] but because only lower temperatures were tested (11.35 – 12.09%).

Table 1 (Part A). Extraction yields obtained from shiitake using individual and combined extraction technologies. Different letters (a-g) denote significant differences ($P < 0.05$).

Technology	Extraction time (min)	Yield of extracted matter/ 100 g shiitake (% w/w)	Yield of precipitated matter/ 100 g extracted fraction (% w/w)	Yield of precipitated matter/ 100 g shiitake (% w/w)
Hot water extraction (HWE)	15	37.99±5.35 ^{de}	20.29±2.88 ^e	7.71±1.09 ^g
	30	34.58±2.22 ^e	19.76±1.20 ^e	6.83±0.41 ^g
	45	39.96±0.06 ^{de}	21.18±0.30 ^{de}	8.46±0.12 ^g
	60	31.24±0.90 ^e	16.59±0.52 ^{ef}	5.18±0.16 ^g
Ultrasound-assisted extraction (UAE)	15	45.74±1.42 ^d	27.26±0.60 ^d	12.47±0.27 ^{fg}
	30	56.19±2.81 ^c	30.90±0.76 ^{cd}	17.36±0.43 ^e
	45	58.52±2.22 ^c	30.92±1.95 ^{cd}	18.09±1.14 ^e
	60	60.36±1.48 ^c	34.56±3.17 ^c	20.86±1.91 ^e
Subcritical water extraction (SWE)	15	73.21±0.91 ^{ab}	52.88±2.12 ^b	38.71±1.55 ^c
	30	75.31±0.91 ^{ab}	23.16±0.56 ^{de}	17.44±0.42 ^e
	45	70.58±3.82 ^b	26.96±0.99 ^{de}	19.03±0.70 ^e
	60	64.49±2.62 ^{bc}	20.05±1.32 ^e	12.93±0.85 ^f
Steam pressurized extraction (SPE)	20	37.96±0.16 ^{de}	11.42±0.01 ^f	4.34±0.01 ^g
UAE+SWE	60 + 15	81.20±1.06 ^a	68.88±2.33 ^a	55.93±1.89 ^a
SFE+UAE	180 + 60	59.73±0.44 ^c	49.42±2.66 ^b	29.52±1.59 ^d
SFE+SWE	180 + 15	74.95±1.26 ^{ab}	63.54±1.75 ^a	47.62±1.31 ^b

Table 1 (Part B). Polysaccharides and other carbohydrates contents obtained in the fractions extracted from shiitake using individual and combined extraction technologies. Different letters (a-c) denote significant differences ($P < 0.05$). *Calculated by subtracting total polysaccharide levels to the total carbohydrate values from the extracted fractions obtained before polysaccharide precipitation

Technology	Extraction time (min)	Total polysaccharides in extracted fractions (g/100g)	Other carbohydrates in extracted fractions (g/100g)*
Hot water extraction (HWE)	15	5.02±0.71 ^c	9.63
	30	4.56±0.27 ^c	18.49
	45	4.02±0.06 ^c	30.09
	60	4.78±0.15 ^c	41.79
Ultrasound-assisted extraction (UAE)	15	8.59±1.41 ^{bc}	13.74
	30	9.37±2.92 ^{bc}	21.04
	45	7.76±1.31 ^c	29.73
	60	10.14±2.46 ^{bc}	36.50
Subcritical water extraction (SWE)	15	15.54±2.18 ^{ab}	12.85
	30	6.80±0.94 ^c	31.64
	45	7.93±1.07 ^c	32.51
	60	5.92±0.67 ^c	27.79
Steam pressurized extraction (SPE)	20	5.13±0.01 ^c	10.16
UAE+SWE	60 + 15	21.14±1.96 ^a	37.97
SFE+UAE	180 + 60	14.46±1.97 ^b	34.46
SFE+SWE	180 + 15	19.88±1.23 ^{ab}	39.27

After the extraction, the obtained fractions were submitted to precipitation to generate polysaccharide-enriched extracts (Figure 1). Results indicated that the HWE fractions contained less material susceptible of ethanol precipitation compared with UAE fractions and particularly the SWE fraction obtained after 15 min extraction. This fraction contained the highest polysaccharide levels yielding 15.5 g/100g SWE fraction, levels that were higher than those obtained using other advanced technologies such as UAE after 60 min (10.1 g/100g UAE fraction) or e.g. microwave-assisted extraction (MAE) (10.5 g/100 g MAE fraction) (Chapter 3, Manuscript 1).

Total β -D-glucan content of shiitake and all generated extracts was determined using a commonly used enzymatic method [41]. The initial raw powder contained 29% (w/w) β -D-glucans representing the 69% of the total carbohydrates quantified being chitins in lower concentrations (5.2%). Both values were in concordance with several works (Chapter 3, Manuscript 3; Chapter 4, Manuscript 1) [42] although they were slightly higher than others [7, 43]. HWE extracted lower β -D-glucan amounts than UAE or SWE except for the SWEs carried out during 60 min (Figure 2a).

In fact, subcritical water extractions longer than 15 min were detrimental to get β -D-glucan-enriched fractions, opposite to results obtained after UAE extractions. In the latter case, 60 min extraction yielded fractions with β -D-glucan concentrations similar to those obtained with only 15 min SWEs.

Moreover, HWE extracted from 6 to 9% of the chitins present in shiitake mushrooms and similarly UAE extracted 5-15%. Only SWE at 15 min showed the highest recovery (35%). However, since chitins are completely insoluble in water, high recoveries were not expected. The extracted compounds might be derivative products from the chitin hydrolysis or depolymerization induced by high temperatures, pressures and/or ultrasounds as suggested in previous works [23]. SWEs displayed a significant decrease in chitin yield (Figure 2b) similar to those recorded for β -D-glucans. It might indicate that extraction time was excessive and therefore the filters from the extraction cell were blocked and no proper extraction was made. However, it might also suggest that the excessive time at 200°C induced hydrolysis and degradation of these two kinds of polysaccharides into lower molecular weight products that were not precipitated as polysaccharides in the analyzed extracts.

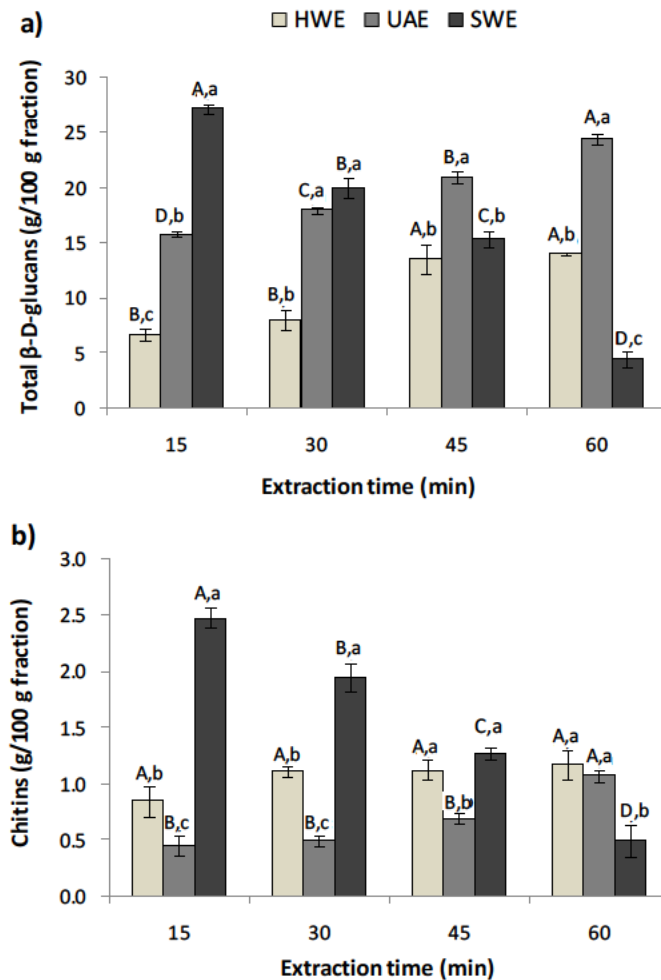


Figure 2. Total β -D-glucan (a) and chitin (b) content in the fractions obtained after HWE, UAE and SWE from shiitake mushrooms. Different letters denote significant statistical differences ($P < 0.05$) between fractions obtained by the same extraction technology at different times (A-D) and obtained by different extraction technologies at the same time (a-c).

In order to test whether the different utilized extraction methodologies showed certain structure selectivity, the β -D-glucan contents were also evaluated using two additional methods developed to detect (1 \rightarrow 3)- or (1 \rightarrow 3),(1 \rightarrow 6)- β -D-glucans [7, 34].

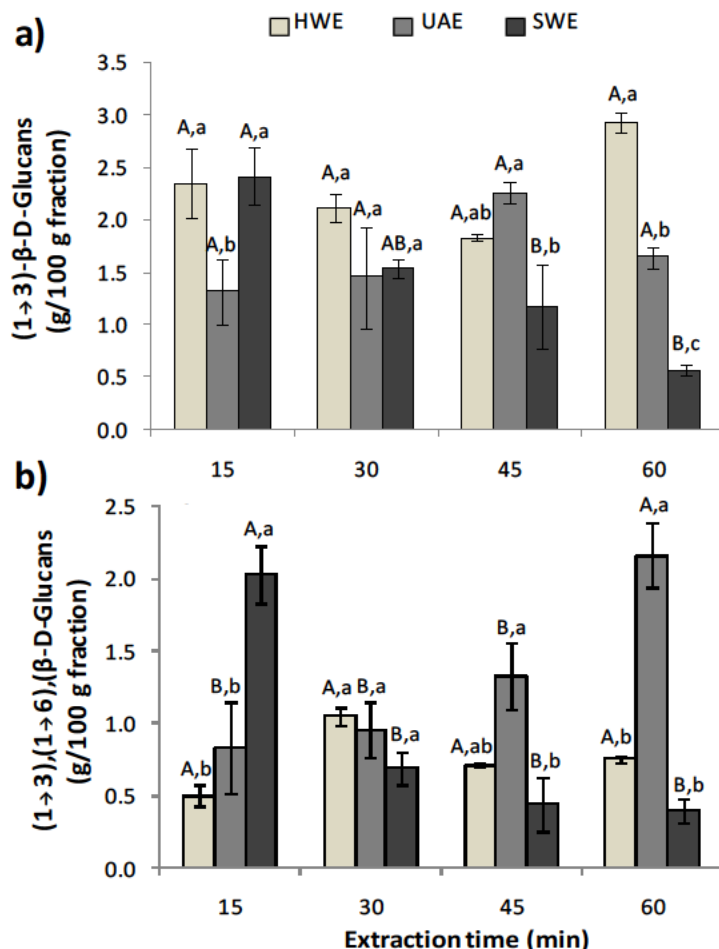


Figure 3. Content of (a) (1→3)-β-D-glucans and (b) (1→3),(1→6)-β-D-glucans in the fractions obtained after HWE, UAE and SWE from shiitake mushrooms. Different letters denote significant statistical differences ($P < 0.05$) between fractions obtained by the same extraction technology at different times (A-B) and obtained by different extraction technologies at the same time (a-c).

The amounts of extracted β-D-glucans were slightly different depending on the methodology utilized e.g. after 15 min, HWE and SWE yielded fractions with similar (1→3)-β-D-glucan concentrations (Figure 3a), however, in the SWE fraction 84% of them were pointed as (1→3),(1→6)-β-D-glucans while the HWE fraction

apparently contained only 21% of β -D-glucans with (1 \rightarrow 6) (Figure 3b). Similarly, after 60 min extraction, UAE fractions showed lower (1 \rightarrow 3)- β -D-glucans than HWE fractions but most of them were detected as (1 \rightarrow 3),(1 \rightarrow 6)- β -D-glucans, reaching levels similar to SWE fractions extracted for 15 min. These results suggested that HWE was extracting more linear glucans than SWE or UAE as linear β -D-glucans are usually bound by (1 \rightarrow 3)- β -linkages while SWE or UAE extracted more branched (1 \rightarrow 3),(1 \rightarrow 6)- β -D-glucans. However, branched glucans are more soluble and therefore they are easily extractable while the linear (1 \rightarrow 3)- β -D-glucans remain firmly attached to the fungal cell wall, requiring stronger extraction methods. For this reason, SWE or UAE were expected to extract more linear glucans than milder treatments (HWE). An explanation for this could be that the (1 \rightarrow 3)- β -D-glucans obtained with HWE presented lower molecular weight being consequently more soluble than those glucans extracted with SWE or UAE (as noticed with HPSEC, see later).

Moreover, a quantitative mismatch was also noticed between the values obtained with the enzymatic method calculating the total β -D-glucan content and the fluorimetric/colorimetric determinations. For instance, SWE (15 min) fractions contained 27.1 g/100g total β -D-glucans (Figure 2) but only 9% of them exhibited fluorescence indicating the presence of β -(1 \rightarrow 3) linkage (Figure 3a) and 7% of them were detected by the colorimetric method as (1 \rightarrow 3), (1 \rightarrow 6)- β -D-glucans (Figure 3b). But, according to previous publications most of the β -D-glucans present in shiitake mushrooms showed β -(1 \rightarrow 3),(1 \rightarrow 6) structures such as for instance lentinan [7], thus, these determined percentages were too low for a main component. Moreover, the SWE fraction obtained after 15 min contained only 15.5 g/100 g polysaccharides (Table 1) and 2.5 g of them were pointed as chitins (Figure 2b). These observations might indicate that selected extraction technologies were extracting more polysaccharides than noticed or that the enzymatic determination might overestimate the total β -D-glucan concentration. Other reports also noticed significantly lower β -D-glucans values in *L. edodes* when fluorimetric methods were utilized compared to the enzymatic procedure [44]. Therefore, the use of indirect colorimetric/fluorimetric determinations should never be considered (in complex mixtures as these extracts) as a precise determination method since their sensitivity might be influenced by the

presence of many interference compounds as suggested for the fluorimetric determination (Chapter 3, Manuscript 1). Thus, in order to define the fraction compositions more precise analytical tools were utilized.

Combining extraction methodologies

Individual extractions indicated that SWE carried out during 15 min and UAE during 60 min were adequate to extract more β -D-glucans than either HWE or SPE methods. However, shiitake contained approx. 34.3% polysaccharides and they were not all extracted. Thus, combination of both technologies UAE+SWE were tested to improve the β -D-glucan content of obtained fractions. Moreover, a pretreatment of the shiitake raw material with supercritical CO₂ was also tested because on the one hand, CO₂ generates an acid environment that might enhance cell structures breakdown and on the other hand, it extract lipid compounds that are within the mushroom dry matter (chitins and β -D-glucans). The latter consequence combined with the high pressure used during SFE might alter cell wall structure facilitating its disruption and the extraction of structural polysaccharides. Therefore, pretreated (SFE) material was submitted to UAE or SWE selecting the optimal extraction times.

The three tested combinations of extracting methods showed very high yields (Table 1), almost doubling those obtained with individual extractions, particularly UAE+SWE. However, not all the extracted material precipitated afterwards and they were not all polysaccharides. Combination of UAE+SWE showed results similar to SFE+SWE, apparently both pre-treatments facilitate the subsequent SWE extracting approx. 31% more polysaccharides than only SWE. SFE pre-treatment also improved approx. 41% the polysaccharide levels obtained in UAE fractions although they still contained less than UAE+SWE or SFE+SWE fractions. The combinations were more effective than SPE methods (5.1%) or other extraction technologies used to obtain polysaccharides-enriched fractions such as MAE (10.5%) (Chapter 3, Manuscript 1).

Apparently, UAE+SWE or SFE+SWE extracted almost all the carbohydrates present in the mushroom (Table 2) and according to the enzymatic protocol they also managed to extract all the β -D-glucans yielding fractions containing approx. 35% β -D-glucans. However, the results from the fluorimetric method indicated that shiitake

contained 10.4% (1→3)- β -D-glucans and the combination of methodologies extracted slightly less compounds yielding fractions with up to 8% (1→3)- β -D-glucans. The combination SFE+UAE was in this case more adequate to concentrate this type of compounds on fractions including up to 9% (1→3)- β -D-glucans where one third of them were (1→3),(1→6)- β -D-glucans. In fact, the three combinations extracted similar concentrations of (1→3),(1→6)- β -D-glucans but they all generated fractions that contained more β -D-glucans than individual extractions. The differences between the selected combinations were more pronounced in their capacity to extract chitin-derivatives compounds. UAE+SWE extracted almost 83% of the chitins detected in the mushroom yielding fractions with more than 5% chitin-derivatives. SFE+SWE were less effective and significantly different than SFE+UAE that extracted even less derivatives. Comparing to other technologies, chitins were present in MAE extracts in approx. 2% [22], therefore, the pretreatment with SFE or UAE particularly before SWE seemed to be a more interesting protocol to extract β -D-glucans and soluble chitins-derivatives than application of individual extraction methodologies, probably because they both facilitate the disruption of the material for a subsequent subcritical water extraction.

However, similar quantitative discrepancies between the enzymatic and the fluorimetric determinations were detected as noticed within the fractions obtained with individual extractions. Thus, the enzymatic method was carried out using 100% starch and schizophyllan as respectively (1→4),(1→6)- α and (1→3),(1→6)- β -D-glucan standards and mixed as 50% starch:sea sand, 50% starch:schizophyllan and 50% schizophyllan:sea sand (w/w). Results indicated that, when only β -D-glucans were present in the mixtures, quantitative determinations were accurate (only 7% error) but, the amount of α -D-glucans was underestimated particularly when mixed with schizophyllan inducing a β -D-glucan overestimation (approx. 14.2%) since the amount of β -D-glucans is calculated by subtracting the α -D-glucan levels to the total glucan concentration. Therefore, the enzymatic, fluorimetric or colorimetric methods should be only used for preliminary assessment and more precise determinations were carried out in order to identify the compounds present in the obtained fractions.

Table 2 (Part A). Carbohydrates determined in shiitake powder and the fractions obtained by SPE and combined technologies (UAE+SWE, SFE+UAE and SFE+SWE). Different letters (a-d) denote significant differences ($P < 0.05$) between values of the same column.

Sample	Total carbohydrates		Total β -D-glucans	
	g / 100 g fraction	g / 100 g shiitake	g / 100 g fraction	g / 100 g shiitake
Shiitake powder	-	42.10 \pm 1.17 ^a	-	28.85 \pm 0.43 ^a
SPE method	15.29 \pm 3.33 ^b	5.80 \pm 1.26 ^c	6.32 \pm 0.29 ^b	2.40 \pm 0.11 ^d
UAE+SWE	59.11 \pm 1.22 ^a	47.99 \pm 0.99 ^a	35.97 \pm 0.43 ^a	29.21 \pm 0.35 ^a
SFE+UAE	48.92 \pm 3.43 ^a	29.22 \pm 2.05 ^b	32.17 \pm 0.28 ^a	19.22 \pm 0.17 ^c
SFE+SWE	59.15 \pm 5.23 ^a	44.33 \pm 3.91 ^a	34.82 \pm 0.32 ^a	26.10 \pm 0.24 ^b

Table 2 (Part B). Carbohydrates determined in shiitake powder and the fractions obtained by SPE and combined technologies (UAE+SWE, SFE+UAE and SFE+SWE). Different letters (a-d) denote significant differences ($P < 0.05$) between values of the same column.

Sample	(1 \rightarrow 3)- β -D-glucans		(1 \rightarrow 3),(1 \rightarrow 6)- β -D-glucans		Chitins	
	g / 100 g fraction	g / 100 g shiitake	g / 100 g fraction	g / 100 g shiitake	g / 100 g fraction	g / 100 g shiitake
Shiitake powder	-	10.40 \pm 0.22 ^a	-	3.22 \pm 0.01 ^a	-	5.19 \pm 0.13 ^a
SPE method	2.94 \pm 0.01 ^b	1.12 \pm 0.01 ^c	0.50 \pm 0.01 ^b	0.19 \pm 0.01 ^c	1.07 \pm 0.09 ^d	0.41 \pm 0.03 ^e
UAE+SWE	8.33 \pm 0.18 ^a	6.76 \pm 0.15 ^b	2.50 \pm 0.32 ^a	2.03 \pm 0.26 ^b	5.30 \pm 0.18 ^a	4.30 \pm 0.15 ^b
SFE+UAE	9.09 \pm 0.94 ^a	5.43 \pm 0.56 ^b	3.03 \pm 0.24 ^a	1.81 \pm 0.14 ^b	3.86 \pm 0.05 ^c	2.31 \pm 0.03 ^d
SFE+SWE	8.12 \pm 0.66 ^a	6.09 \pm 0.49 ^b	2.51 \pm 0.14 ^a	1.88 \pm 0.10 ^b	4.76 \pm 0.14 ^b	3.57 \pm 0.10 ^c

Chemical composition of the obtained fractions

The monosaccharide composition of the polysaccharide-enriched extracts obtained with individual and combined extraction technologies was analyzed by GC-MS. Results pointed out the presence of mannose, galactose, and glucose, being the latter in high levels (Table 3). Higher extraction periods led to higher amounts of glucose in HWE extracts, while the glucose content of UAE extracts (and of the other

two monosaccharides) was almost independent of the extraction time. The glucose levels were in concordance with β -D-glucan determinations, e.g. SWE extracted higher β -D-glucan amounts at 15 min than at longer extraction times as well as higher glucose levels. UAE+SWE extracts showed higher glucose levels than UAE but similar to SWE extracts suggesting that probably with the first extraction (UAE) only part of the glucans were extracted and in the second extraction an approximately 20% more was further extracted to yield the same values as with SWE alone. Heteropolymers including mannose and galactose in their structure are usually easily water-soluble since they are weakly attached to other molecules from the fungal matrix [45]. HWE extracts obtained with short extraction times showed higher levels of mannose than longer extractions. On the contrary, SWE extracts (15 min) contained lower mannose and lower galactose contents, being glucose more concentrated suggesting that SWE might show certain specificity to isolate β -D-glucans than other heteropolymers. UAE extracts showed higher levels of mannose and galactose and lower glucose levels than SWE (15 min), suggesting a more heterogeneous composition than the SWE extract. The use of SFE pretreatment slightly enhanced the β -D-glucan selectivity noticed for SWE since SFE+SWE extracts showed a slightly higher percentage of glucose and lower galactose levels. This combination was more specific than UAE+SWE where more mannose and less glucose were noticed.

The different monosaccharide composition noticed depending on the extraction methodology utilized suggested the presence of different polymers, therefore, the extracts were also analyzed by HPSEC. Results showed heterogeneous elution profiles (Supplementary figure S1) confirming the presence of polysaccharides with different molecular mass. Similar profiles were observed for HWE extracts obtained at different extraction times (Figure S1a) UAE extracts obtained after 60 min extractions were also slightly different than the other extracts obtained at shorter extraction times (Figure S1b) but differences were more pronounced for SWE extracts. Those fractions obtained after 15 min extraction showed a peak with maximum at approx. 57 min indicating the presence of medium molecular weight compounds (Figure S1c). With increasing extraction times, their profile shifted to other peaks with maxima approx. at 60 min indicating the presence

of lower molecular weight compounds and therefore, suggesting degradation provoked by the large extraction method. UAE+SWE and SFE+SWE extracts showed a profile similar to SWE extracts while in SFE+UAE extracts seemed to increase the amount of high molecular weight compounds (R.T. approx. 45 min) compared to UAE extracts. The high molecular weight compounds noticed in UAE-related extracts were lacking in SWE extracts suggesting that the polymers extracted with SWE showed a more narrow size distribution (medium molecular weight) while UAE-related extracts contained a more heterogeneous size distribution.

Table 3. Monosaccharide composition (%) of the precipitated extracts obtained from shiitake by the different individual and combined technologies.

Technology	Extraction time (min)	Monosaccharides (%)		
		Mannose	Glucose	Galactose
Hot water extraction (HWE)	15	21.9	68.6	9.5
	30	16.1	71.2	12.7
	45	9.3	81.5	9.2
	60	9.2	81.3	9.5
Ultrasound-assisted extraction (UAE)	15	22.3	62.0	15.7
	30	22.2	61.8	16.0
	45	22.8	62.9	14.3
	60	24.3	63.1	12.6
Subcritical water extraction (SWE)	15	10.4	83.9	5.7
	30	12.2	82.1	5.7
	45	25.0	68.5	6.5
	60	26.5	63.9	9.6
Steam Pressurized extraction (SPE)	20	13.6	74.0	12.4
UAE+SWE	60+15	12.2	82.8	5.0
SFE+UAE	180+60	11.4	81.4	7.2
SFE+SWE	180+15	10.0	85.3	4.7

Moreover, to identify the compounds present in polysaccharide-enriched extracts from the individual and combined extraction methodologies, NMR studies were carried out and compared with literature data (Supplementary figure S2). The extracts contained a mixture of polysaccharides, including α - and β -D-glucans and a

heteropolymer composed of mannose and galactose [2, 45, 46]. Although samples were precipitated with cold ethanol, trehalose signals were observed (δ 92.9/4.82 ppm and 92.6/4.81 ppm). Trehalose is a disaccharide, also known as mycose, and that is commonly found in large amounts in mushrooms. This sugar was also detected in *Pleurotus ostreatus* extracts obtained by SWE in previous works [22] and it could be removed from the polysaccharide fractions after performing dialysis (data not shown).

Intense signals relative to C-1 of α -D-Glcp (δ 99.3-99.5/4.97-4.98) and to C-3 *O*-substituted (δ 82.8-82.9/3.54-3.55) were observed, suggesting the presence of (1 \rightarrow 3)- α -D-glucan structures, that were also previously reported and isolated from other mushrooms such as *Fomitopsis betulina* [46]. Moreover, the extracts showed intense ^{13}C signals arising at 102.4-103.7 ppm and ^1H signals at 4.14-4.44 ppm confirming the presence of C-1 of glucans in β -configuration [47], as well as signals at the range of δ 85.8-87.0 (^{13}C) and at δ 3.23-3.39 (^1H) related to C-3 *O*-substituted. In addition, inverted signals at δ 68.1-68.5/3.89-3.99 and δ 68.1-68.5/3.37-3.50 confirmed CH_2 *O*-substitution of the same units, being in the concordance with the scientific literature, where the mainly studied glucan from shiitake mushrooms is a branched (1 \rightarrow 3),(1 \rightarrow 6)- β -D-glucan.

Small intensity signals of α -D-Galp were observed in almost all spectra at 98.4-98.6/4.63-4.65 ppm as well as signals of β -D-Manp at δ 101.1-101.5/4.82-4.84 ppm, being in concordance with the results of monosaccharide composition and suggesting the existence of a heteropolymer consisting of galactose and mannose.

According to these results, it was possible to observe that the polysaccharide-enriched fractions obtained from different extraction methods presented similar polysaccharide composition: (1 \rightarrow 3)- α -D-glucans, (1 \rightarrow 3)(1 \rightarrow 6)- β -D-glucans, and a heteropolymer composed of mannose and galactose. The main difference among them seemed to be the proportion of each polysaccharide extracted.

Biological activities of the obtained extracts

The production of β -D-glucan-enriched fractions should be encouraged for the production of functional foods because fungal β -D-glucans are biologically active molecules with many interesting biological activities. However, their beneficial

properties can be influenced by their extraction procedure since some of them might modify the tridimensional structure of the polymer [10]. Therefore, the hypocholesterolemic and immunomodulatory activities of the precipitated extracts with the highest β -D-glucan contents were selected to evaluate whether after the extraction treatments, they still maintain their biological activities.

Table 4. HMGCR inhibitory activity (%) of the obtained precipitated extracts obtained by HWE during 60 min (HWE60') and combination of advanced methodologies. Different letters (a-c) denote significant differences ($P < 0.05$) between samples.

Sample	HMGCR inhibition (%)
HWE60'	42.52 \pm 0.01 ^c
UAE+SWE	84.41 \pm 6.98 ^b
SFE+UAE	89.03 \pm 0.48 ^{ab}
SFE+SWE	87.32 \pm 1.89 ^{ab}
Pravastatin	99.21 \pm 0.36 ^a

Water soluble extracts from several mushrooms including soluble β -D-glucans were able to inhibit the key enzyme of the cholesterol metabolism [48], therefore, obtained extracts were tested as HMGCR (3-hydroxy-3-methylglutaryl coenzyme A reductase) inhibitors. Results indicated that SFE+UAE and SFE+SWE extracts showed inhibitory capacity similar to 0.5 mg/mL pravastatin, the statin utilized as positive control (Table 4). The UAE+SWE extract exhibited a slightly lower capacity. The combination of these two extraction methodologies seemed to generate extracts with lower inhibitors concentration or to be slightly detrimental for the structure of the HMGCR inhibitors but still, it was better combination than HWE where half of the inhibitory activity was lost. Gil-Ramirez et al. (2013) [49] studied the HMGCR inhibitory activity of SWE extracts obtained from *L. edodes* and indicated that extracts obtained at 150 °C (5 cycles 5 min) showed less inhibitory activity than similar extracts obtained at 25°C probably because the inhibitors were thermal sensitive. However, SFE+SWE or UAE+SWE extracts still retained very high inhibitory activity. Perhaps, the pre-treatment with UAE or SFE prior to SWE protected/modulated the inhibitors structure making them more active or less

susceptible to thermal degradation or perhaps, the *L. edodes* strain was different than the one utilized in the previous work as differences between varieties or cultivation methods seemed to influence their inhibitory properties too [49].

The immunomodulatory activities of the extracts obtained with combined methodologies were also tested as their capacity to reduce the secretion of pro-inflammatory cytokines in macrophages differentiated from THP-1 human monocytes cell line. The preliminary experiments to assess the extract cytotoxicity indicated that when applied up to 200 µg/mL viability of THP-1 macrophages was not affected (data not shown). Thus, the immunomodulatory activity was tested in two subtoxic concentrations (100 and 200 µg/mL). The THP-1 macrophages stimulated with LPS (positive control) exhibited a significant release of the three pro-inflammatory cytokines studied (1295 pg/mL TNF- α , 4586 pg/mL IL-1 β and 1837 pg/mL IL-6) compared to non-stimulated cells (negative control) (Figure 4). Addition of the extracts obtained with combined methodologies significantly reduced the amount of TNF- α liberated in the media. When applied at the highest tested concentration a 24 to 32% reduction in TNF- α was noticed (down to 883 pg/mL in the case of SFE+UAE), however, the extract obtained after 60 min HWE did not effectively modulate any response. SFE+UAE seemed to be more effective because it induced the mentioned TNF- α reduction and it was the only extract inducing a 25% reduction (3462 pg/mL) in IL-1 β secretion when applied at 200 µg/mL. The IL-6 release was also inhibited approx. 25% by SFE+UAE and SFE+SWE (1420 and 1343 pg/mL, respectively) extracts being the latter also significantly effective when applied at lower concentration. However, SWE extracts obtained from *L. edodes* at 50 °C (5 cycles 5 min) reduced approx. 90% the release of IL-6, IL-1 β and 20% TNF- α while if the temperature was higher (200 °C) only a slight reduction in IL-6 was noticed [50]. Thus, the temperature utilized in the production of the SFE+SWE extract could have impaired their beneficial immunomodulatory properties. Thus, SFE+UAE was a more adequate combination of methodologies to maintain the immunomodulatory properties since its extract could reduce the liberation of all

pro-inflammatory cytokines tested but, their activity might be influenced by the temperature utilized.

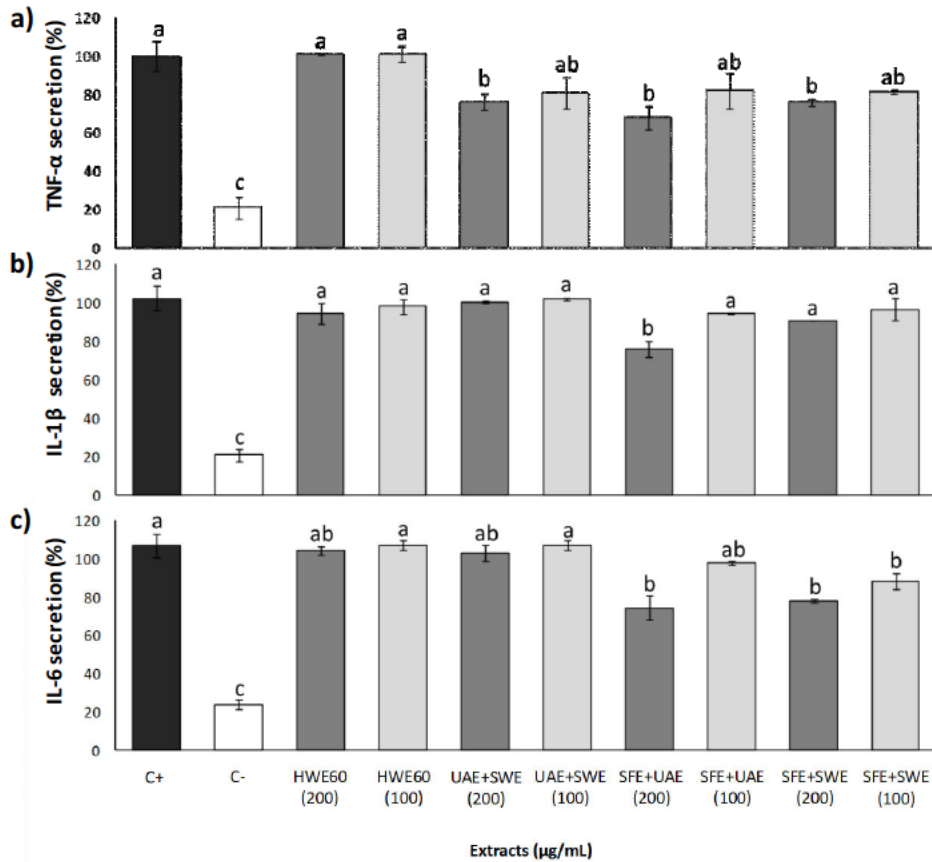


Figure 4. Levels of a) TNF- α , b) IL-1 β and c) IL-6 secreted by THP-1/M activated with LPS in presence of HWE (60 min), UAE+SWE, SFE+UAE and SFE+SWE extracts. Positive control: cells stimulated with LPS but in absence of extract. Negative control: non LPS-activated cells. Different letters (a-c) denote significant differences ($P < 0.05$) between samples.

Conclusion

In conclusion, submission of shiitake mushrooms to UAE or SFE followed by UAE or SWE were more effective methods to obtain β -D-glucan-enriched fractions than individual UAE or SWE extractions. The generated fractions contained

approx. 20% polysaccharides although according to enzymatic determinations they included approx. 34% β -D-glucans. Fluorimetric/colorimetric methods pointed out lower amounts of (1 \rightarrow 3) and (1 \rightarrow 3),(1 \rightarrow 6) linked β -D-glucans. These discrepancies might be caused because they were determined by indirect rough methods that should be considered as preliminary estimations. More accurate analytical methods indicated that UAE fractions contained more heterogenic composition (more mannose and galactose and less glucose contents) and polymers of higher molecular weight than SWE (15 min) or SFE+SWE fractions. UEA-related extracts (e.g. 60 min or SFE+UAE, etc.) also extracted lower chitin-derivative contents than SWE-related extracts (15 min, UAE+SWE, SFE+SWE, etc.). However, the main differences among the fractions seemed to be the extracted polysaccharides ratio since they all contained (1 \rightarrow 3)- α -D-glucans, (1 \rightarrow 3),(1 \rightarrow 6)- β -D-glucans, and heteropolymers composed of mannose and galactose. Moreover, the obtained fractions might be of interest to design functional foods with hypocholesterolemic properties since they showed high HMGCR inhibitory activity, even higher than other reported extracts obtained at high temperatures similar to those used in SFE+SWE or SFE+UAE [49]. But, the immunomodulatory properties of the extracted β -D-glucans might be compromised because although they were still able to reduce secretion of TNF- α , IL-1 β and IL-6 in macrophage cell lines, only the SFE+UAE fractions managed to reduce them approx. 20% while other extracts obtained using SWE at lower temperatures were reported more effective [50].

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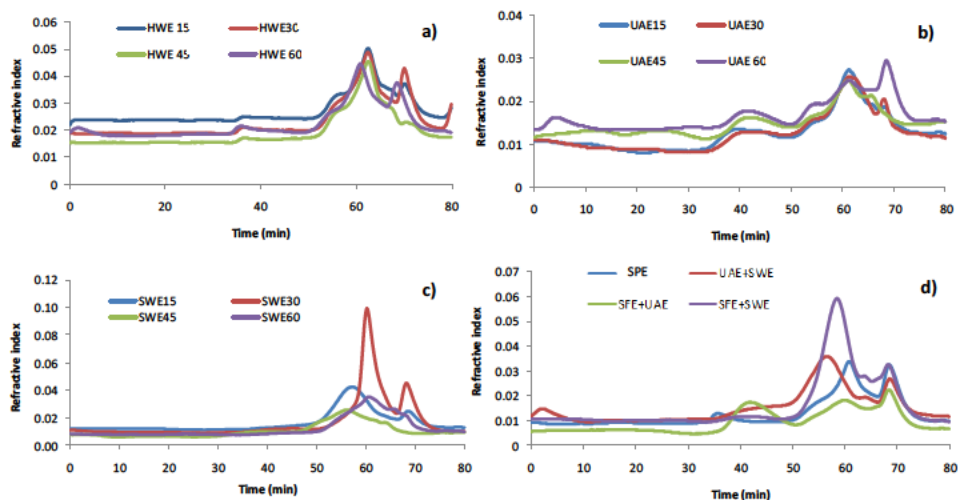
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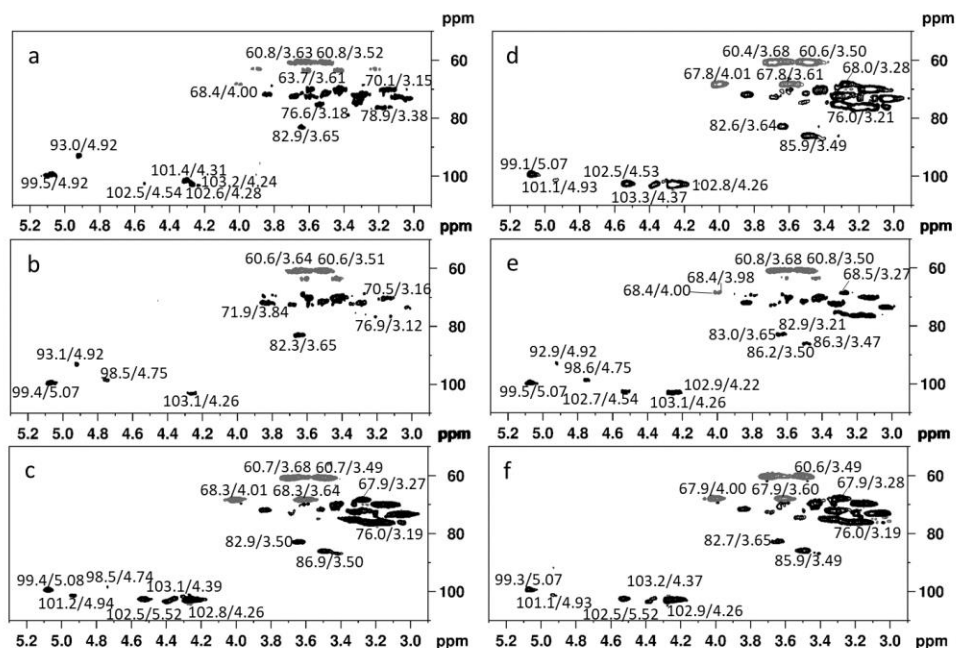
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Supplementary information



Supplementary Figure 1. HPSEC elution profiles of a) HWE, b) UAE and c) SWE fractions obtained at different extraction times (15 to 60 min) and d) SPE and combined fractions.



Supplementary Figure 2. HSQC-DEPT35 NMR spectra of extracts obtained after a) HWE 60 min, b) UAE 60 min, c) SWE 15 min, d) UAE+SWE, e) SFE+UAE and f) SFE+SWE. Experiments were performed in DMSO at 70 °C (chemical shifts are expressed in δ ppm).

Manuscript 3

Production of a β -D-glucan-rich extract from shiitake mushrooms (*Lentinula edodes*) by an extraction/microfiltration/reverse osmosis (nanofiltration) process

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Abstract

A pilot-scale process combining extraction of shiitake mushroom (*Lentinula edodes*) powder in water (98 °C, 1 h), cross-flow microfiltration and reverse osmosis (nanofiltration) was performed to obtain β -D-glucan-rich extracts. Suspensions (45-80 L) obtained after 3 extractions were clarified by microfiltration reducing their turbidity to < 1 NTU. Membrane flux was completely recovered after filtration. One of the clarified extracts was concentrated (to 6-7 L) by reverse osmosis (Nanomax95) and the other two by nanofiltration (Nanomax50 and ALNF99-2517). Different physicochemical parameters (permeate flux, total soluble substances, total suspended particles and electrical conductivity) were monitored during filtration and the bioactive compounds present in the obtained fractions (β -D-glucans, total carbohydrates, chitins, eritadenine, lenthionine, ergosterol) were analyzed. The more adequate membrane for shiitake extract concentration was Nanomax50 because it showed higher filtration flux and higher values of bioactive compounds in the obtained extract than the extracts obtained with the other two membranes.

Industrial relevance: This work describes a pilot-scale procedure for obtaining β -D-glucan-rich extracts from *Lentinula edodes* (shiitake mushrooms). The extracts might be used in novel functional foods due to their high content in hypocholesterolemic compounds. The process combines extraction with boiling water, cross-flow membrane clarification and reverse osmosis/nanofiltration concentration of β -D-glucans. The procedure is scalable to industrial level.

Introduction

β -D-glucans are polysaccharides consisting of D-glucose units linked by β -glycosidic bonds which can be found in several natural sources such as yeasts, bacteria, algae, cereals and mushrooms [1, 2]. They have attracted attention due to their biological activities, including immune-modulating, anti-tumour, antioxidant and anti-inflammatory properties [3-6]. Furthermore, several studies indicated that β -D-glucans are also able to lower serum cholesterol and blood glucose [7-10].

Different extraction procedures at lab, pilot or industrial scale have been described and adapted depending on the source and nature of the β -D-glucans and most of them are based on hot water extractions. However, sometimes their proper solubilization requires more aggressive conditions: alkaline or acidic mediums, ultrasound, microwave and enzymes aids or temperatures higher than 100 °C and these treatments might also contribute to their partial degradation or modification of their native structure altering their biological activities [11-14].

After extraction, several purification steps are required to concentrate β -D-glucans, usually by removing other substances. One of the most used, simple and effective procedure for separation of β -D-glucans from other molecules (particularly from low molecular mass compounds) is their precipitation with ethanol [13], although other solvents such as ammonium sulfate or acetone solutions have also been used [15, 16]. Other purification techniques are column-based fractionation procedures such as size exclusion, anion-exchange or affinity chromatography [17-19], but they are useful mainly for preparative separation at lab scale. For purification of larger extract volumes, membrane separation processes such as ultrafiltration and diafiltration have been efficiently used to purify β -D-glucans and other polysaccharides from mushrooms and other food [20-23].

In this study, shiitake mushroom (*Lentinula edodes*) powder was submitted to extraction in boiling water to obtain a β -D-glucan-enriched extract with beneficial properties for cardiovascular health. A double stage cross-flow microfiltration and reverse osmosis/nanofiltration was used to remove suspended particles and to increase β -D-glucan concentration. Filtration was carried out using different membranes and

some physicochemical parameters of the concentrate and permeate streams were evaluated during the filtration (total water soluble substances, turbidity and electrical conductivity) to define the optimal treatment. Furthermore, β -D-glucan content was determined in the obtained extracts and after each purification step as well as other important compounds of shiitake mushrooms with hypocholesterolemic activities such as eritadenine, ergosterol and chitins (as source to generate chitosan).

Materials and methods

Biological material and reagents

Fine powder (particle size < 0.5 mm, moisture < 5%) of *Lentinula edodes* S. (Berkeley) fruiting bodies was purchased from Glucanfeed S.L. (La Rioja, Spain) and stored in darkness at -20 °C until further use.

Hexane (95%), chloroform (HPLC grade), methanol (HPLC grade) and acetonitrile (HPLC grade) were obtained from LAB-SCAN (Gliwice, Poland) and absolute ethanol, sodium carbonate (Na_2CO_3) and sulfuric acid (H_2SO_4), from Panreac (Barcelona, Spain). Potassium hydroxide (KOH), ascorbic acid, 2,6-Di-*tert*-butyl-*p*-cresol (BHT), bovine serum albumin (BSA), Bradford reagent, acetylacetone, *p*-dimethylaminebenzaldehyde, Tris (Trizma®) base, hydrochloric acid (37%), trifluoroacetic acid (99%), phenol, as well, as hexadecane, ergosterol (95%), D-glucose and D-glucosamine hydrochloride were purchased from Sigma-Aldrich Quimica (Madrid, Spain). β -glucan Assay Kit Megazyme® was acquired from Biocon (Barcelona, Spain), D-eritadenine (90%) from Sy Syntchem UG & Co. KG (Felsberg, Germany) and lenthionine (80%) from Cymit (Barcelona, Spain). All other reagents and solvents used in this study were of analytical grade. MilliQ-grade water was produced in a MilliQ® Integral 3 purification system (Merck Millipore, Billerica, USA); demineralized water with electrical conductivity of 7-8 $\mu\text{S}/\text{cm}$ was obtained by a reverse osmosis (RO) unit (Genius 300, Filtec Depuradoras, Girona, Spain).

Pilot scale solid/liquid extraction unit

Large amounts of shiitake extracts (45-80 L) were prepared using a pilot-scale solid/liquid extraction unit, provided with a 30L extraction vessel and a thermostatic system, allowing temperatures maintenance from 30 to 99 °C. A Nylon mesh strainer bag with a mean pore size of 30 μm was used to hold the suspension in the extraction vessel. Homogenization of the suspension and intensification of the matter transfer from the solid to the liquid phases were carried out by mechanical agitation of the suspension inside the strainer bag.

Pilot scale pressure-driven crossflow membrane unit

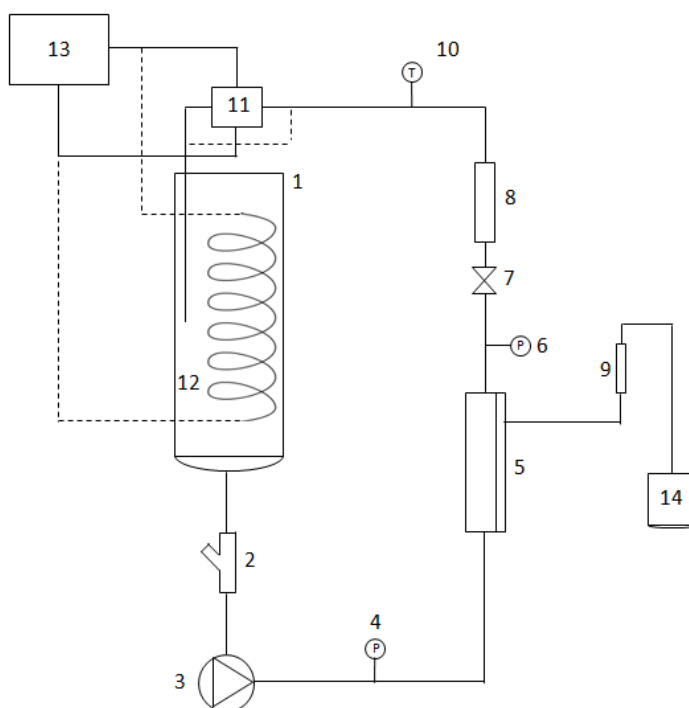


Figure 1. Scheme of multifunctional pilot pressure-driven cross-flow membrane device: (1) feed tank, (2) strainer, (3) pump, (4) inlet pressure gauge, (5) membrane housing, (6) outlet pressure gauge, (7) pressure control valve, (8) concentrate flowmeter, (9) permeate flowmeter, (10) thermometer, (11) plates and frames heat exchanger, (12) tubular spiral wound heat exchanger, (13) chiller and (14) permeate recipient vessel.

All treatments for clarification and concentration of the shiitake extracts were carried out by a self-designed multifunctional pilot pressure-driven crossflow membrane unit (Figure 1), provided by a 20 L feed tank (1), 0.25 mm mesh stainless still Y-type strainer (2), variable flow (0-2500 L/h)/high pressure (up to 50 bar) membrane pump (Hydra-Cell G-25, Wanner Engineering Inc., Minneapolis, USA) (3), inlet pressure gauge (0-6 or 0-60 bar) (4), membrane housing (5), outlet pressure gauge (0-6 or 0-60 bar) (6), needle pressure control valve (7), online concentrate rotary flowmeter (8), online permeate rotary flowmeter (9), online thermometer (10), online plates and frames (11) or immersed tubular spiral wound (12) heat exchangers, chiller (13) and permeate recipient vessel (14). Depending on the used membranes and housings, the unit has a dead volume of 6 to 7 L. All tubing's, vessels and parts of the measurement instruments which were in contact with the fluid were from AISA 316 and 316L stainless still. Pump membranes were of food grade ethylene propylene diene monomer (EPDM) and the mobile parts of the tubing's, from silicone.

Lab-scale extraction of β -D-glucans from shiitake mushroom powder

Temperatures of 60 and 98 °C and extraction times of 1, 2 and 3 h were tested at lab scale, according to the following procedure: mushroom powder (10 g) was placed into 0.5 L round flasks and 300 mL MilliQ-grade water was added to each of them (33.3 g/L). The mixtures were heated at the respective temperatures and stirred in a thermal magnetic plate (with reflux for extractions at 98 °C). Furthermore, the same amounts and ratio of mushroom powder and water were autoclaved at 120 °C during 20 min. The soluble fractions were clarified by centrifugation (7000 rpm, 7 min) and freeze-dried. In addition, extractions were performed again and polysaccharides were precipitated by adding 3 volumes of ethanol, mixing vigorously and keeping the mixtures overnight at 4 °C. Later on, the samples were centrifuged (10,000 rpm, 15 min) and the precipitates lyophilized. Moreover, different substrate to solvent ratios were tested apart from 33.3 g/L such as 50, 66.7, 133.3 and 200 g/L at 98 °C during 1 h. All extractions were carried out in duplicate.

Pilot-scale extraction of β -D-glucans from shiitake mushroom powder

After optimization of the extraction parameters at lab scale, three series of extractions (ES1, ES2 and ES3) were carried out at pilot scale in order to adequate some extraction parameters, such as processing time, suspended particle load, β -D-glucan concentration and batch volumes to the capacities and limitations of the membrane separation equipment. The studied parameters are shown in Table 1.

Table 1. Amounts of solvent (water), substrate (shiitake powder), substrate to solvent ratio and number of extraction batches used in the three extraction series (ES1, ES2, ES3) of β -D-glucans from shiitake mushrooms at pilot plant conditions.

Extraction series	Water used for extraction	Shiitake powder used for extraction	Substrate to solvent ratio	Number of extraction batches
	(L)	(kg)	(g/L)	(-)
ES1	60	1.5	50.0	2
ES2	90	2.0	66.7	3
ES3	120	1.8	60.0	4

Extraction series 1 (ES1) reproduced directly the optimal conditions obtained at lab scale. ES2 and ES3 were carried out to improve the extraction efficiency and adequate the extract volumes to the transformation capacity of the microfiltration (MF) unit.

In all cases, demineralized water (30 L) was heated up to 85 °C. Shiitake powder (Table 1) was introduced into the Nylon mesh strainer bag and immersed into the hot water with intense agitation in order to disperse the solid phase into the liquid phase and inhibit the action of polyphenol oxidases naturally present in Shiitake mushrooms. Then, the suspensions were heated up to 98 °C and maintained at this temperature for 1 h. Extractions were ended by draining the liquid phase from the extraction chamber. The insoluble particles that remained in the strainer bag were frozen and freeze-dried. The cloudy extract was left to settle overnight at 4 °C and for ES2 and ES3, the clear phase was separated by racking and stored at 4 °C for the next

fine clarification by MF (ES1 was submitted to MF without racking). The sediments were centrifuged and the solid phase was freeze-dried and added to the insoluble solids. The supernatant was added to the settled extract and submitted to the next MF process.

Fine clarification of shiitake extracts by cross-flow membrane microfiltration (MF)

Fine clarification of the cold settled shiitake extracts was carried out by the above described pressure-driven membrane unit (Figure 1), set at the low-pressure mode. In this case the equipment includes a tubular spiral wound heat exchanger (12) (immersed into the feed tank (1)), inlet and outlet pressure gauges (4 and 6), sensitive in the interval of 0-6 bar and a multichannel ceramic membrane from CeraMem Corporation (Waltham, MA, USA) (Table 2). Prior to use, the membrane was conditioned by washing with demineralized water. Initial (L_p^0) and post-regeneration (L_p^R) membrane hydraulic permeability was determined by plotting the water flux values at different transmembrane pressures (P_{TM}) at temperature of 20 ± 2 °C. Pressure was fixed by varying the inlet flow rate at completely open pressure control valve. The same measurements were carried out with the shiitake extract at the same operating conditions.

Table 2. Technical characteristics of the used membranes (MF: microfiltration, NF: nanofiltration, RO: reverse osmosis).

Membrane designation	Housing model	Nominal pore size (μm)	NaCl rejection (%)	MgSO ₄ rejection (%)	Filtration surface (m^2)	Membrane material	Mode of operation
LM-0500-M	not available	0.5	-	-	0.13	mixed oxides	MF
Nanomax95	Helicon-RO4	-	94	97	0.37	polyamide	RO
Nanomax50	Helicon-RO4	-	65	96	0.37	polyamide	NF
ALNF99-2517	M2.5-PN64	-	-	$\geq 98\%$	1.0	composite	NF

Fine clarification was carried out by filtration of the extracts in a concentration mode at 0.7 bar constant P_{TM} and 20 °C. When 5 L of permeate were collected, 5 L of the initial cold settled extract were added into the feed tank all over the treatments. Concentration volumetric factors (F_C) for each treatment were calculated as: $F_C = V_{initial}/V_{final}$, where $V_{initial}$ was the initial volume of the extract and V_{final} was the final volume of the corresponding concentrate. Filtration was stopped when the whole volume of extract was processed. The highly dense concentrate (approx. 7 L) was discarded. Samples (5 mL) were collected during filtration from both permeate and concentrate streams and analyzed for their content of total soluble substances (TSS or °Brix) and turbidity.

Concentration of shiitake extracts by nanofiltration (NF) / reverse osmosis (RO)

Concentration of the fine clarified shiitake extracts was carried out by the already described pressure-driven membrane unit (Figure 1) set at the high-pressure mode. In this case, plates and frames heat exchanger (11) and high pressure (0-60 bar) inlet and outlet pressure gauges were used. Three spiral wound membranes, Nanomax95 and Nanomax50 from Millipore (Bedford, MA, USA) and ALNF99-2517 from Alfa Laval (Lund, Sweden) (Table 2) were tested. Prior to use, the membranes were conditioned by washing with demineralised water. Initial (L_p^0) and post-regeneration (L_p^R) membrane hydraulic permeability was determined as described earlier for the MF membrane. The feed flow rates (Q_F) for Nanomax95 and Nanomax50 membranes were set at 600 and 800 L/h respectively, whereas those for the Alfa Laval's membrane was 1100 L/h. Pressure was fixed by appropriate adjusting of the pressure control valve. The same measurements were carried out with the Shiitake extract, at the same operating conditions.

Processing was carried out by filtration of the extracts in a concentration mode at 24-29 bar P_{TM} and 20 °C. Operational P_{TM} were determined from the corresponding plots of filtration flux (J) = $f(P_{TM})$. In general, they were selected as the highest value from the linear response of each membrane to the corresponding extract. Thus, the extracts from the ES1, ES2 and ES3 series were concentrated by

Nanomax95, Nanomax50 and ALNF99-2517 at P_{TM} of respectively 24, 29 and 25 bar. When 5 L of permeate were collected, 5 L of clarified extract were added into the feed tank, all over the treatments. F_C 's for each treatment were calculated as described already for the microfiltration process. Filtration was stopped when the whole volume of extract was processed. The concentrate was drained from the equipment, freeze-dried and stored at 4 °C for further analysis as well as aliquots of 5 L of the 3 permeates. Samples of 5 mL were collected during filtration from both, permeate and concentrate streams and analyzed for their content of total soluble substances (TSS), turbidity, pH and electrical conductivity.

After filtration, membranes were cleaned by consecutive washings with water at room temperature for 15 min, followed by water at 45 °C for 15 min and chemical regeneration with 0.5 M NaOH solution at 50 °C during 60 min for the MF membrane and 0.1 M NaOH solution at 45 °C for 60 min for the NF and RO membranes. Final washing with water until both rejection and permeate streams became neutral (pH 7) at room temperature was carried out for all membranes before conservation.

Retention coefficients (R) were calculated for each measured parameter, according to $R = (1 - C_p/C_f) \cdot 100 (\%)$, where C_p and C_f are the concentration of each measured parameter in the permeate and the feed streams, respectively.

Physicochemical analyses

Total soluble substances (TSS) of the extracts were measured directly by hand-held refractometer Atago in the interval of 0 to 32% (°Brix). Total suspended particles (TSP) were estimated indirectly by measuring turbidity with a TN100 infrared turbidimeter (Thermo-Fisher Scientific, Spain), attuned for measurements in the interval of 0 to 800 NTU (nephelometric turbidity units). Samples with turbidities higher than 800 NTU were diluted appropriately with water to enter in this interval and values were calculated according to the number of dilutions. Electrical conductivity was determined by direct measurement of samples by a GLP 32 model conductivity meter from Crison Instruments (Barcelona, Spain).

Chemical composition analyses

β -D-glucan content was measured using a mushroom and yeast specific β -D-glucan kit (β -glucan Assay Kit Megazyme®) following the instructions of the user's manual and as described in Palanisamy et al. (2014) [9].

Total carbohydrate content was determined by the phenol-sulphuric acid method, adapted from Dubois et al. (1956) [24], as described in Fox & Robyt (1991) [25].

Chitin content was determined by a colorimetric method based on Smiderle et al. (2017) [14]. For this, the sample (5 mg) were hydrolyzed with 6 M HCl at 100 °C for 2 h and adjusted to pH 10.0 after cooling down. The hydrolyzed sample (250 μ L) was used for the colorimetric method according to Rementeria et al. (1991) [26]. Samples were read at 530 nm using an Evolution 600 UV-VIS (Thermo Fisher Scientific, Spain) spectrophotometer and glucosamine hydrochloride was used to prepare the standard curve.

Sterols were extracted from shiitake powder and its soluble and insoluble parts, using the equipment and following the procedure described by Gil-Ramirez et al. (2013) [27]. Ergosterol was used as standard to develop and validate the GC method and hexadecane (10% v/v) as internal standard.

Eritadenine was extracted from the samples following the procedure of Afrin et al. (2016) [28] with some modifications. Briefly, samples (1 g) were mixed with 10 mL of 60% ethanol (v/v) and stirred for 2 min. The mixtures were centrifuged (15 min, 7000 rpm, 10 °C) and the supernatants were collected by decantation. Afterwards, aliquots of 10 mL of 60% ethanol (v/v) were again added for a second extraction and both supernatants were pooled together and submitted to vacuum filtration. The filtrate was concentrated on a rotary vacuum evaporator at 60 °C until dryness. Identification and quantification of eritadenine were carried out using a C18 Spherisorb ODS2, 250 x 4 mm i.d. analytical column with a 5 μ m particle size (Waters, Missisagua, Ontario, Canada), coupled to an HPLC system (Pro-Star 330, Varian, Madrid, Spain) with photodiode array detector (PAD). Samples were dissolved in water:acetonitrile (98:2, v/v 1% TFA) used as mobile phase (5 mg/mL)

and aliquots of 10 μL were injected and developed at constant flow (0.5 mL/min). Eritadenine was identified and quantified at 260 nm using a commercial standard showing its characteristic UV-spectra and retention time (11.6 min).

Lenthionine extraction was carried out according to the procedure of Hiraide et al. (2010) [29] with slight modifications. Basically, samples were mixed with 0.2 M Tris-HCl buffer pH 8.0 (50 mg/mL) and stirred for 1 h. Afterwards, 0.5 mL methanol was added and the mixture was stirred and centrifuged (14000 rpm, 5 min). Supernatant was collected and pooled together with the others obtained after repeating this step three times. Later on, MilliQ water (2.5 mL) was added to the supernatants and filtered through a PVDF filter (0.45 μm). The solution was introduced into a 50 mg ODS Sep-Pack cartridge (Waters, Missisagua, Ontario, Canada) preconditioned according to the manufacturer instructions. The cartridge was washed with 1 mL of 30% methanol (v/v) and lenthionine was eluted with 1 mL of 65% methanol (v/v). Finally, the eluate (50 μL) was injected into an HPLC-PAD system (same column and equipment than previously described for eritadenine analysis) to identify and quantify lenthionine (retention time 10.4 min) at 230 nm using an isocratic mobile phase of 65% methanol, constant flow (0.7 mL/min) and 45 $^{\circ}\text{C}$ column temperature. Commercial lenthionine was used as a reference substance.

Statistical analyses

Differences were evaluated at 95% confidence level ($P \leq 0.05$) using a one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison test. Statistical analysis was performed using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA).

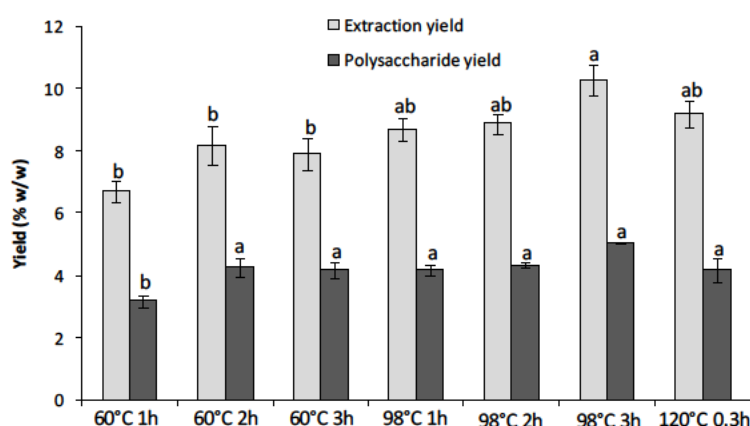
Results and discussion

Extraction of β -D-glucans at lab scale

Lab scale preliminary β -D-glucan extractions were carried out to figure out optimal extraction temperature, time and substrate to solvent ratio. Results indicated that the extraction yields of total polysaccharides were rather similar under most of

the studied conditions (Figure 2a). Extraction at 98 °C for 3 h showed the highest yield (5% w/w) being similar to those found by Smiderle et al. (2017) [14] and Anguilo-Aguayo et al. (2017) [11] using respectively microwaves, pressure and heat at 50 °C during 5 min and ultrasounds during 15 min. However, stronger heat exposure (> 2 h at 98 °C and only 20 min at 120 °C, Figure 2b) was detrimental for β -D-glucan content, suggesting that 98 °C and 1 or 2h were more suited for the extraction of soluble β -D-glucans.

a)



b)

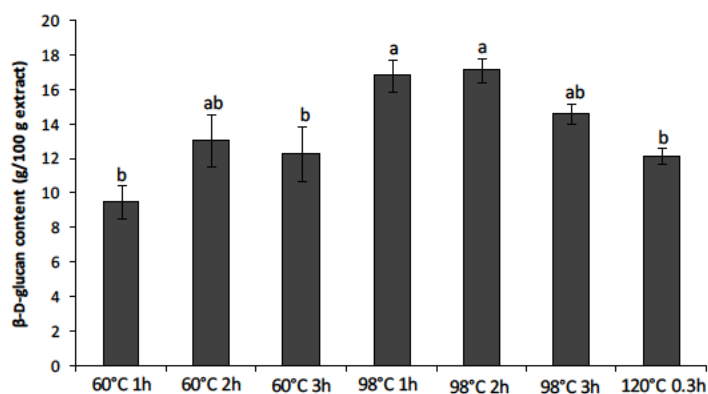


Figure 2. Dry matter, total polysaccharide (a) and β -D-glucan (b) extraction yields obtained after extraction of Shiitake powder at lab scale at different temperatures and time (% w/w of initial dry matter). Different letters (a-b) between values of the same series denote significant differences ($P < 0.05$).

In addition, different substrate to solvent ratios (33, 50, 67, 133 and 200 g/L) were tested and no significant differences between extraction yields were found (data not shown) but ratios over 50 g/L hindered the separation of the liquid extract from the insoluble fraction because of the clogging of the Nylon bag cloth. Therefore, a ratio of 50 g/L substrate to solvent was selected for the first extraction series (ES1) at pilot scale.

Extraction of β -D-glucans at pilot scale

Pilot-scale extractions were carried out to define processing time, suspended particle load, β -D-glucan concentration and batch volumes prior to subsequent membrane separation processes.

Firstly, direct up scaling of the optimal lab conditions were tested: substrate to solvent ratio was 1.5 kg shiitake powder/30 L water/batch and they were macerated at 98 °C during 1 h (ES1, Table 1). Two extraction batches were carried out at these conditions and a total volume of 45 L of extract (E1) was obtained containing 1.9% TSS, 17.8% β -D-glucans and 1407 NTU turbidity (Table 3). Then, the obtained β -D-glucan-rich extract was clarified by MF in one batch for a reasonable time of < 8 h, at relatively low P_{TM} (0.7 bar) (Figure 4) and without any technical or operational problems. These results suggested that improving of the β -D-glucan yield and increasing of the processing time were possible.

Table 3. Whole extract volume, turbidity (before and after cold clarification), total soluble substances (TSS) and β -D-glucan content (dry weight) of the shiitake mushroom extracts (E1, E2 and E3) obtained after the three extraction series (ES1, ES2, ES3) at pilot plant conditions.

Extraction series	Whole extract volume	Turbidity before fine clarification	Turbidity after fine clarification	TSS content	β -D-glucan content
	(L)	(NTU)	(NTU)	(%)	(%)
ES1	45	1407	0.28	1.9	17.8
ES2	55	1832	0.28	4.2	13.2
ES3	80	1213	0.64	2.9	13.4

In the second extraction series (ES2) an increase of the substrate to solvent ratio of 50 g/L to 66.7 g/L and the number of extraction batches from 2 to 3 was carried out. Nevertheless, these changes led to an important decrease of the permeate flux (Figure 4) and increase of the TSP content (Table 3), which was detrimental for the MF equipment as some part of the suspended particles tended to decant and obstruct the equipment piping and connections, leading to breaking of the process and a need for dilution of the extract to reestablish the filtration process. However, the membrane resisted this heavy operational conditions and the whole volume of shiitake extract was processed in only one batch, without any physical or chemical cleaning of the membrane. These outcomes evidenced that substrate to solvent ratio of 66.7 was too high for further treatments, but that the volume of extract might be still increased.

Thus, the last extraction series (ES3) was carried out at a substrate to solvent ratio of 60 g/L (Table 1). Four extraction batches of 30 L were sequentially carried out and 80 L of total extract (E3) were produced, containing 1213 NTU turbidity, 2.9% TSS and 13.4% β -D-glucans (Table 3). Filtration of an extract with such physicochemical characteristics allowed the establishment of very good filtration flux of 70-60 L/(h·m²) (Figure 4) at relatively low P_{TM} (0.7 bar) in the MF process and to transform 80 L of extract in one MF batch (without intermediate discharge of concentrate) and without any technical or operational problem. Nevertheless, the transformation of 80 L of extract would require a change of 2 turn's regime of staff to cover an interval of 20 h of working time.

Results of the β -D-glucans analysis showed that the extract obtained at the lowest substrate to solvent ratio (E1) had the highest concentration of soluble β -D-glucans, 17.8% (Figure 3). This value was very similar to the obtained at the same conditions at lab scale. Nevertheless, in all cases, the content of soluble β -D-glucans was considerably lower than those of the initial raw shiitake powder (SP) (29%) and the insoluble material (R1-R3) (36-41%), indicating the existence of a considerable quantity of β -D-glucans that could not be extracted by the studied extraction conditions. These results suggest that other techniques, such as alkaline or ultrasound- or microwave-assisted or steam explosion extraction should be considered if higher

yields of soluble β -D-glucans are required. However, taking into account costs for investments and scaling to pilot equipment for production of larger extract amounts, simple solid/liquid extraction with boiling water is still one of the most technically and economically viable alternatives.

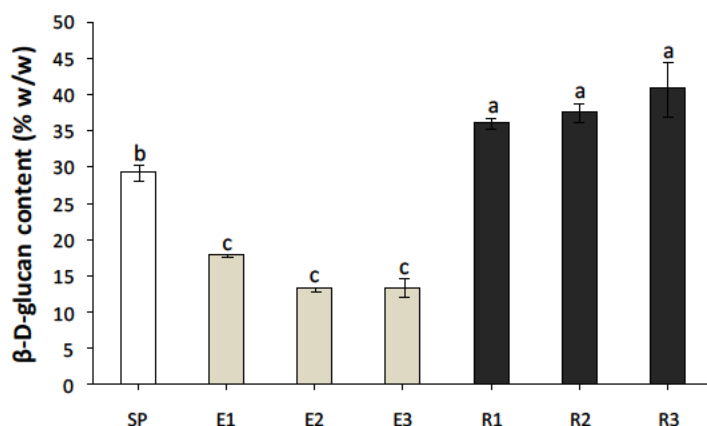


Figure 3. β -D-glucan content (% w/w) of raw Shiitake mushroom powder (SP), soluble extracts (E1, E2, E3) and insoluble fractions (R1, R2, R3) from the three studied extraction series (ES1, ES2, ES3). Different letters (a-c) denote significant differences ($P < 0.05$) between values of the same series.

Aliquots of soluble and non-soluble extracts were submitted to centrifugation to observe differences in the content of β -D-glucans, but no significant differences were found (data not shown) so this centrifugation step was not included in the procedure at pilot scale.

Clarification of β -D-glucan-rich extracts by cross-flow microfiltration

Effect of the physicochemical characteristics of the extracts on the main parameters of operation

Fine clarification of the cold-settled shiitake extracts was carried out by cross-flow microfiltration, at the low-pressure mode (P_{TM} 0.7 bar, 20 °C). The kinetics analysis of the permeate fluxes of the three studied Shiitake extracts (E1, E2

and E3) (Figure 4) indicated that the highest filtration flux was recorded during extract clarification with 2.9% TSS (E3) (Table 3, Figure 5c).

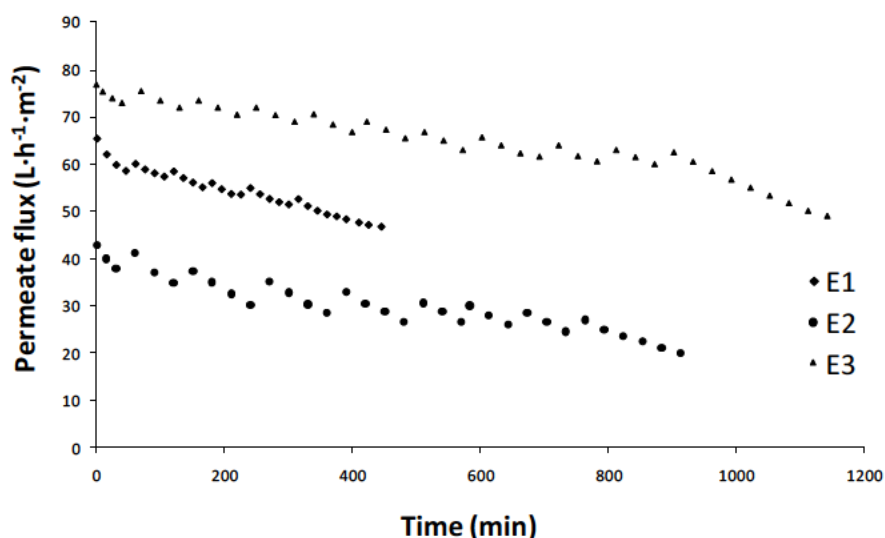


Figure 4. Permeate flux kinetics ($\text{L} / (\text{h} \cdot \text{m}^2)$) during cross-flow microfiltration of the studied Shiitake extracts, E1, E2 and E3.

In this case, 80 L extract were concentrated to 7 L (F_C of 11.4) and 73 L filtrate was recovered, without breaks or any operational problem in less than 20 h. The treatment started at $77 \text{ L}/(\text{h} \cdot \text{m}^2)$ and diminished down to $49 \text{ L}/(\text{h} \cdot \text{m}^2)$ at the end indicating a really good filtration flux. It could also be noticed that the extract flux with the highest TSS content (4.2%) (E2) was much lower (Table 3, Figure 5b), comprising the interval of $43\text{--}20 \text{ L}/(\text{h} \cdot \text{m}^2)$. In this case, 55 L were concentrated to 7 L (F_C of 7.9) for 14 h and 48 L of filtrate was recovered. The problem with this extract was not only the lowest filtration flux, but also some difficulties related to partial obstruction of the equipment tubing with insoluble particles and subsequent breaks of the process for purging the equipment tubing to reestablish filtration. Extract E1 with the lowest TSS content (1.9%) was filtered without operational problems, but showed lower filtration flux ($65\text{--}47 \text{ L}/(\text{h} \cdot \text{m}^2)$) than E3, effect which could be explained with the higher TSP (turbidity) load (Table 3). As filtration was carried out by concentration of an initial volume of 20 L of each extract, an effect of concentrate dilution was produced at each addition of 5 L of unfiltered extract (Figure 5), resulting in certain increase of the filtration fluxes (Figure 4). In all cases, filtration

fluxes were inversely proportional to the increase of the TSS contents of the extracts (Figure 5) and to the increase of the total suspended particle concentration (turbidity) in the concentrate streams (Figure 6). The first effect was more evident between extracts than during filtration of each of them (Figure 5), whereas the second was most easily appreciable during filtration than between extracts (Figure 6).

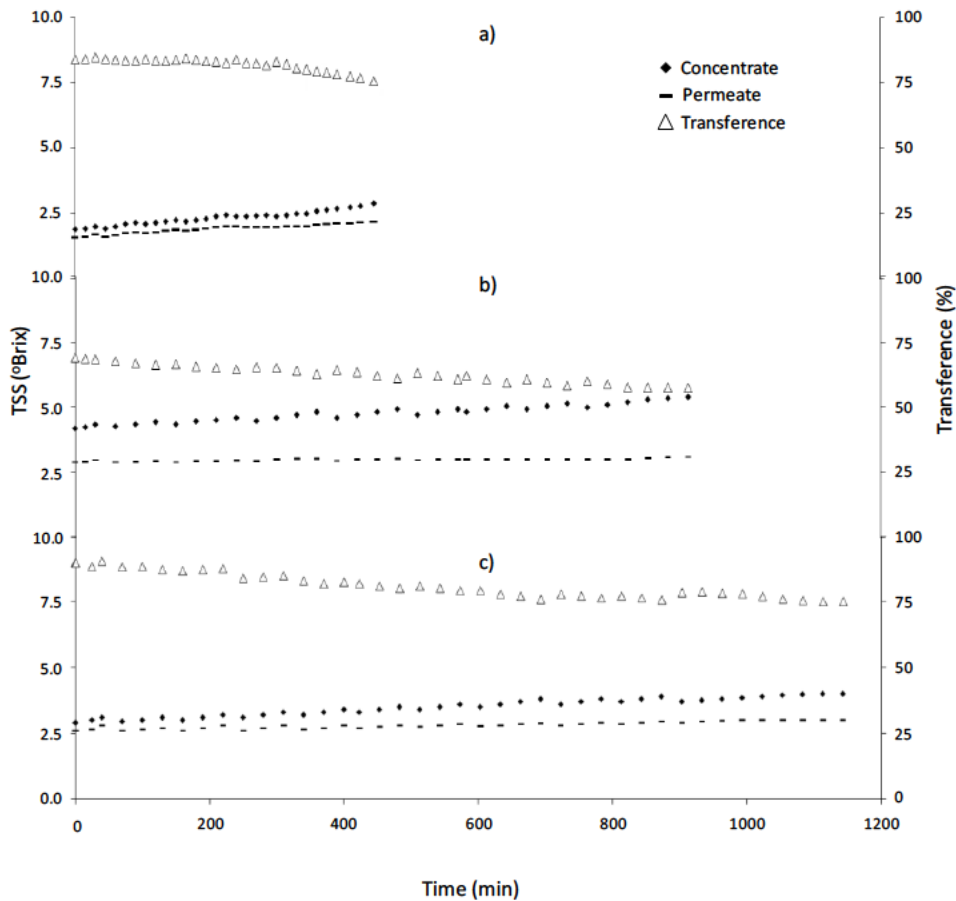


Figure 5. Distribution of total soluble substances (TSS) in the concentrate and permeate flows and transference of TSS throughout the membrane during cross-flow microfiltration of the studied Shiitake extracts E1 (a), E2 (b) and E3 (c). (♦ are TSS_{conc}, total soluble substances in the concentrate; - are TSS_{perm}, total soluble substances in the permeate; Δ are TSS_{transf}, transference of total soluble substances through the corresponding membrane).

The observed permeate flux kinetics of the studied extracts showed faster flux declines at the beginning of the treatments and establishment of steady fluxes afterwards. This effect is usually related to the buildup of a secondary fine-particle/colloidal layer on the membrane surface and depends mostly of the TSP and the colloidal part of the TSS.

When TSS content of the extracts was relatively low (1.9-2.9% for E1 and E3, respectively), the transference of TSS throughout the membrane was high (between 90 and 75%), but not entirely, leading to retention of some soluble compounds in the part of the concentrate (Figure 5). In the case of the extract with the highest TSS load (4.2%) (E2) these values were considerably lower, 60-55%. These findings indicate that the filtration flux is inversely related to the TSS load and suggest that, although the 0.5 μm pore size membrane was rated as a microfiltration membrane, it rather acted as an ultrafiltration membrane.

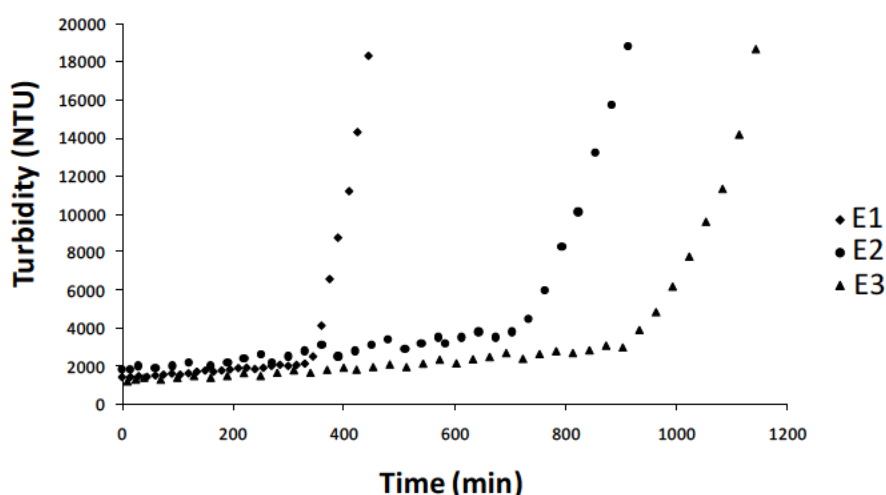


Figure 6. Kinetics of turbidity (NTU) in the concentrate flow during microfiltration of the studied shiitake extracts E1, E2 and E3.

The concentrates obtained after the three microfiltration treatments contained the finest fraction of insoluble β -D-glucans. They could be added to the insoluble fraction left after the extraction of the soluble β -D-glucans, but they were discarded because of their high microbial load.

With respect to the TSP load of the extracts, the turbidities of the three cold-settled extracts were very high (Table 3), comprising the interval of 1213 (E3) and 1832 NTU (E2). The operational problems appeared during filtration of the E2 suggested that turbidities higher than 1800 were inappropriate for this process and should be avoided.

As filtrations were carried out by addition of 5 L fractions of unfiltered extracts (to the 15 L concentrates), only small increases of turbidity were observed during these periods (Figure 6), while the concentration of the last 15 L of each extract resulted in an exponential turbidity increase. That is why the filtration of the 45 L of E1 entered in this exponential phase earlier than the 55 and 80 L of respectively E2 and E3.

The results from Figures 4-6 suggest that for an optimal cross-flow microfiltration, TSS of the Shiitake extracts should be around 3%, but not higher than 3.5% (substrate to solvent ratio around 60 g/L or lower than 65 g/L) and that it was possible to clarify volumes higher than 80 L/0.1 m² (800 L/m²) of membrane filtration surface in one concentration batch (without discharge of the concentrate). But probably, the most important feature of these experiments was the complete recovery (100%) of the membrane permeability after standard regeneration with 0.5 M NaOH solution, which revealed the high physical and chemical resistance of this membrane in the clarification of Shiitake extracts with high loads of suspended particles.

Effect of the cross-flow microfiltration on the quality of the soluble β -D-glucan-rich extracts and recovery of the rests of insoluble β -D-glucans

The effect of the 0.5 μ m pore size membrane on the clarification of the three studied shiitake extracts (E1, E2 and E3) was excellent, giving turbidities in the narrow interval of 0.3-0.6 NTU (Table 3). These low turbidities values indicated the complete separation of the soluble from the insoluble β -D-glucans and warranted the microbiological stabilization of the obtained soluble β -D-glucan-rich extracts. In fact, the filtrates were microbiologically stable during several weeks whereas the concentrates developed off-odors in few hours after treatment if they were not frozen.

Nevertheless, the data from Figure 5 also showed that this membrane retained up to 25% of TSS in all of the studied extracts and this retention was directly proportional to the increase of the TSS concentration in the concentrate flow. These results indicated that some parts of the high molecular mass compounds (most probably soluble β -D-glucans) were retained in the suspended particle fraction. Partial recovery of these compounds is possible by the introduction of an additional diafiltration step of the final concentrate or by decreasing the volume concentration factor (F_C) of the process. The first option would lead to an increase of operation time and expenses for elimination of the added water and the second one would decrease the volume of the filtered extract. However, results from the analysis of β -D-glucan content of the studied extracts (Figure 10) showed that the retention of β -D-glucan in the concentrates was insignificant in the three cases.

Concentration of clarified β -D-glucan-rich extracts by reverse osmosis (RO)/nanofiltration (NF)

Effect of the physicochemical characteristics of the extracts on the operation parameters

For concentration of the clarified Shiitake extracts one RO and two NF membranes were tested, using the pressure-driven membrane unit described in Figure 1, set at the high-pressure mode. Thus, 38 L of the clarified E1 extract with 2.2% of TSS were concentrated to 6 L with 7.2% of TSS (Figure 8a) ($F_C = 6.3$) by the polyamide RO (Nanamax95) membrane at P_{TM} of 24 bar (20 °C) for 4.3 h, without any operational problem. During the treatment, the filtration flux dropped from 30 to 18 L/h.m² in an almost lineal mode (Figure 7), which denotes 40% loss of the initial flux.

Then, 48 L of the clarified E2 extract with 3.1% of TSS were concentrated to 6 L with 15.4% of TSS (Figure 8b) ($F_C = 8$) by the polyamide NF (Nanamax50) membrane (0.37 m² of filtration surface) at P_{TM} of 29 bar (20 °C). In this case, a membrane with 65% NaCl rejection (96% MgSO₄ rejection, Table 1) was used and an initial filtration flux of 119 L/(h.m²) was achieved, even the higher TSS load of this

extract (Figure 8b). Nevertheless, this flux dropped to 38 L/h.m² in 1.5 h which means almost 68% loss of the initial flux.

Finally, 74 L of the clarified E3 extract with 3.0% of TSS were concentrated to 7 L with 13.6% of TSS (Figure 8c) ($F_C = 10.6$) with a NF (ALNF99) membrane, at P_{TM} of 26 bar (20 °C). This membrane had a larger filtration surface (1 m²) and >98% rejection of MgSO₄ (Table 1), but an initial filtration flux of 75 L/(h · m²) was achieved, even the TSS load of this extract was lower than the E2 (Figure 8). In this case the loss of filtration flux was smaller (around 65%), which is also due to the lower TSS concentration of the extract.

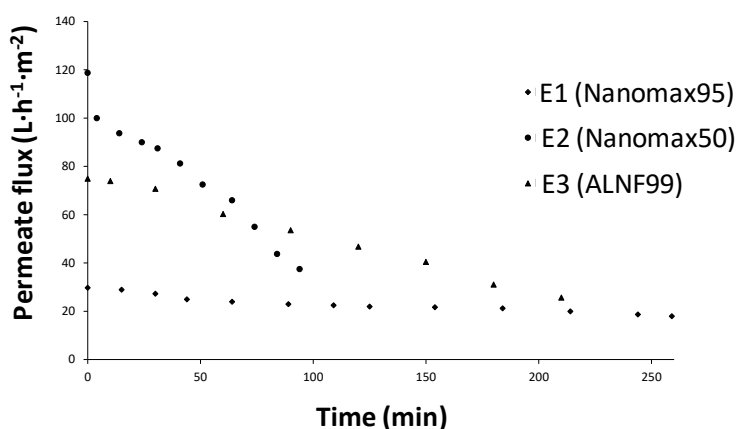


Figure 7. Permeate flux kinetics (L / (h · m²)) during concentration of the clarified E1, E2 and E3 Shiitake extracts by the corresponding to each of them membranes Nanomax95, Nanomax50 and ALNF99.

As it was expected, the obtained results showed that the Nanomax95 reverse osmosis membrane had 2.5 to 4 times lower initial filtration flux than the two nanofiltration membranes, ALNF99 and Nanomax50, respectively, even that the E1 extract had the lowest TSS load. This can be clearly related to the high rejection rate of this membrane and the slightly lower pressure at which the treatment was carried out. The difference found between the initial fluxes of the two nanofiltration membranes were not negligible and should be related to differences in the nature of the materials, as Nanomax50 was a polyamide membrane and ALNF99 was not specified (but not polyamide). In this context, it should be highlighted that the

ALNF99 membrane lost completely its retention capacity after the next 2 months of storage at the conditions specified by the producer. No clear reasons for this failure were found and the use of this membrane was abandoned.

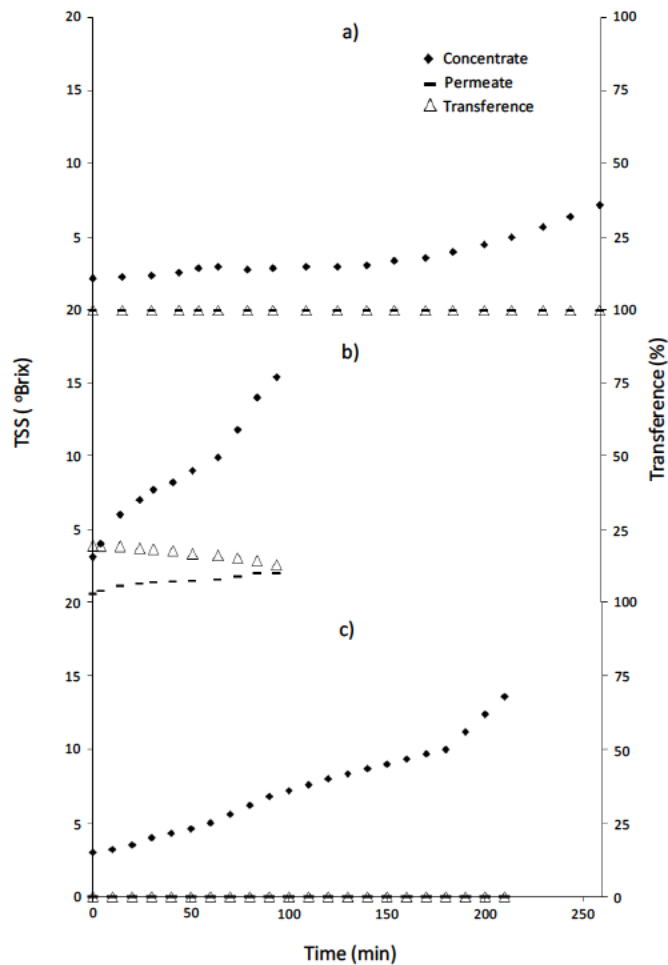


Figure 8. Kinetics of the total soluble substance contents (TSS) during concentration of the clarified E1, E2 and E3 Shiitake extracts by the corresponding to each of them membranes Nanomax95 (a), Nanomax50 (b) and ALNF99 (c) (♦ are TSS_{conc} , total soluble substances in the concentrate; - are TSS_{perm} , total soluble substances in the permeate; Δ are TSS_{transf} , transference of total soluble substances through the corresponding membrane).

Moreover, in all cases, filtration fluxes decreased in linear modes depending directly of the TSS concentration in the concentrate streams (Figures 7 and 8) and this dependence was more pronounced as higher was the initial TSS load of the extracts. On the other hand, there was no zone of steady flux, as it was observed in the case of the clarification treatments (Figure 4), mostly due to the absence of suspended particles and/or colloids in the extracts indicating that there was no evidence for formation of concentration-polarization phenomena in these treatments.

The obtained results showed that the treatments with the studied RO and NF membranes allowed reducing the initial volumes of the fine-clarified extracts to volumes of 6-7 L, completely suitable for direct drying.

Effect of the concentration on the content of soluble β -D-glucans

For evaluation of the effect of concentration of the studied Shiitake extracts a criteria of minimal losses of soluble β -D-glucans and higher removal of other substances was adopted. For this, electrical conductivity was measured during the concentration process. Solution electrical conductivity is basically related to the content of inorganic and organic electrolytes, such as mineral salts, carboxylic acids, some amino acids and peptides, among others.

Non-electrolytes, such as carbohydrates have zero or very low electrical conductivity, thus measurement of this parameter during concentration of carbohydrate solutions may be very indicative for separation of the electrolytes. In addition, it is an easy and fast measurement which can be carried out during the treatment.

The results for electrical conductivity, measured during concentration of the clarified E1, E2 and E3 shiitake extracts (Figure 9) showed that the RO membrane Nanomax95 retained almost completely all electrolytes in the concentrate, i.e. there was no separation from the carbohydrates. The treatment with the NF membrane ALNF99 produced certain transference of electrolytes (up to 5%), mostly at the last concentration period, whereas the Nanomax50 NF membrane showed the highest transfer of electrolytes, with values comprised in the interval of 32 to 25% at the end of concentration.

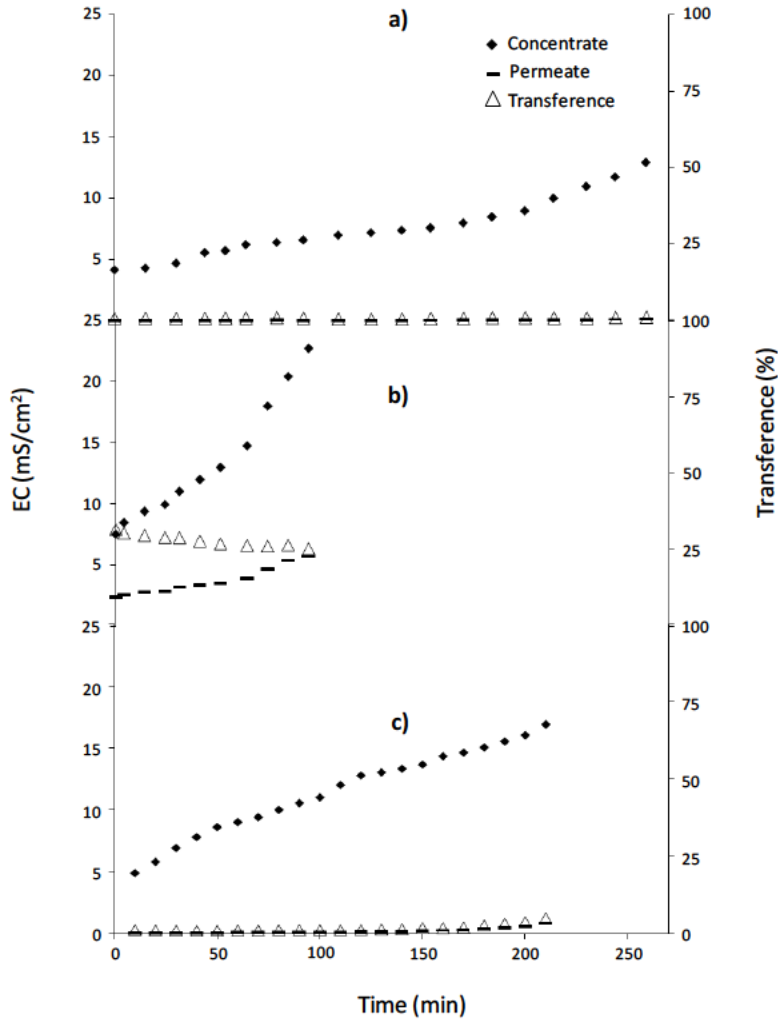


Figure 9. Electrical conductivity (EC) during concentration of the clarified E1, E2 and E3 Shiitake extracts with Nanomax95 (a), Nanomax50 (b) and ALNF99 (c), membranes, respectively. (♦ are TSS_{conc}, total soluble substances in the concentrate; - are TSS_{perm}, total soluble substances in the permeate; △ are TSS_{transf}, transference of total soluble substances through the corresponding membrane).

These results indicate that the Nanomax50 membrane not only concentrated the shiitake β -D-glucans, but also purified them from an important part of the extract electrolytes.

Evaluation of the global processing of β -D-glucan-rich extracts at molecular level

β -D-glucan contents were quantified in the initial cold-settled shiitake extracts (E1, E2 and E3), their corresponding fine clarified extracts (microfiltered permeates, MP1, MP2 and MP3) and concentrates obtained by Nanomax95 (RF1), Nanomax50 (RF2) and ALNF99 (RF3) membranes (Figure 10). Results indicated that, in general, the MF process did not produce any change of β -D-glucan concentration of the most concentrated E2 and E3 extracts (2.9 and 4.2% of TSS), whereas a small but significant loss of 20% was observed in the extract with the lower concentration of TSS (1.9%, E1) (Figure 10). With respect to the concentration treatment, the RO Nanomax95 and the NF ALNF99 membranes had no effect on the β -D-glucan contents of the extracts, whereas the NF Nanomax50 (RF2) produced a low, but significant β -D-glucans enrichment (21%), due basically to the removal of some electrolytes. Having in mind the highest filtration flux obtained by this membrane, it could be suggested that Nanomax50 was the most adequate membrane for this treatment.

Beside β -D-glucans, other bioactive compounds were determined in the obtained extracts, since they also exhibited cardiovascular protective effects such as eritadenine, ergosterol, lenthionine -an organosulfur compound which showed capacity to inhibit platelet aggregation *in vitro* [30] and chitins as source of chitosan derivatives.

The aqueous extracts E1, E2 and E3 contained between 28 and 35% (w/w) of total carbohydrates (TC) (Table 4), amounts relatively low compared to those of the insoluble residues (R1, R2, R3) (48-53%) indicating that relevant amounts of mushroom carbohydrates remained in the solid phase, which could not be extracted with boiling water. This result was already observed particularly for the β -D-glucans (Figure 2) and is extensible for other polysaccharides, such as chitins (Table 4). In general, the MF/NF/RO treatments did not affect significantly the total carbohydrate and chitin contents all over the treatment.

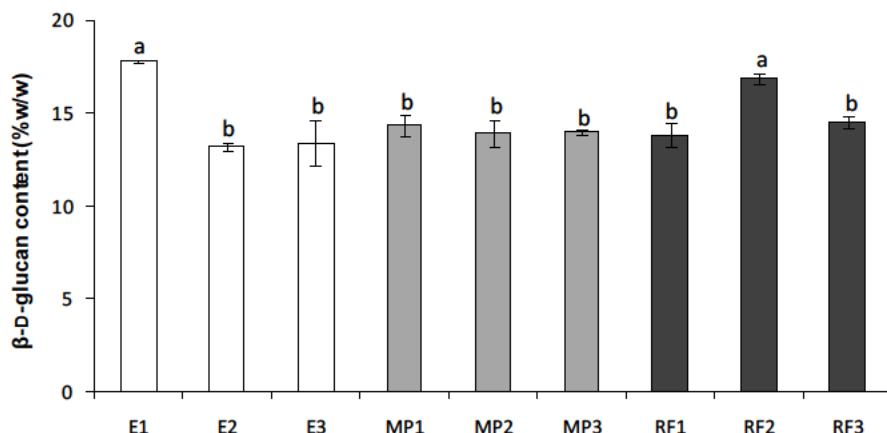


Figure 10. β -D-glucan contents (% w/w) of the three cold-settled (E1, E2 and E3), fine clarified (MP1, MP2 and MP3) and concentrated shiitake extracts by Nanomax95 (RF1), Nanomax50 (RF2) and ALNF99 (RF3) membranes. Different letters (a-b) denote significant differences ($P < 0.05$) between values of the same series.

The highest concentration ratio of total carbohydrates (TC after concentration/TC before concentration) was achieved when Nanomax50 NF membrane was used (1.09), followed by the ALFN99 NF (1.03) and Nanomax95 RO membrane (0.89). Significantly lower amounts of chitins were found in the extracts before and after MF/NF/RO treatments, indicating that only a remaining fraction of low molecular mass and degradation derivatives from chitins were present after filtration. These chitin products were also observed in previous reports [9].

Results also indicated that eritadenine was easily extracted with boiling water and that the MF/NF/RO treatments increased its concentration up to 6.9 mg/g, indicating that this water soluble compound resists high temperatures as previously reported. On the contrary, lenthionine that was present in the shiitake powder before heating was not detected in any of the obtained extracts confirming the results of Shiga et al., 2014[31] indicating that lenthionine suffers thermal degradation above 80 °C.

Ergosterol, a lipophilic compound with hypocholesterolemic effect was mainly found in the insoluble residue at concentrations approx. 5 mg/g (Table 4).

These values were higher than their usual levels in mushroom hyphae, therefore a 2.1 fold concentration ratio was obtained and the insoluble fraction might be considered for compound recovery.

Table 4. Concentration of bioactive compounds (total carbohydrates (TC), chitins (CH), eritadenine (EA), lenthionine (LT) and ergosterol (ER)), measured in Shiitake powder (SP), the water-insoluble fractions (R1, R2, R3) and the cold-settled (E1, E2 and E3), fine clarified (MP1, MP2, MP3) and concentrated by Nanomax95 (RF1), Nanomax50 (RF2) and ALNF99 (RF3) membranes shiitake extracts. Different letters (a-c) denote significant differences ($P < 0.05$) between values of the same series.

	TC	CH	ER	EA	LT
	(% w/w)	(% w/w)	(mg/g)	(mg/g)	(mg/g)
SP	40.67±0.65 ^b	6.03±0.29 ^b	2.40±0.02 ^b	1.44±0.14 ^c	0.15±0.01 ^a
R1	49.03±4.18 ^a	9.08±0.87 ^a	4.81±0.05 ^a	0.57±0.02 ^c	n.d. ^b
R2	48.01±3.09 ^{ab}	9.12±0.46 ^a	5.00±0.48 ^a	1.01±0.03 ^c	n.d. ^b
R3	53.36±1.32 ^a	9.25±0.76 ^a	4.69±0.21 ^a	0.31±0.03 ^c	n.d. ^b
E1	35.06±3.75 ^b	1.85±0.97 ^c	0.75±0.04 ^c	4.49±0.69 ^b	n.d. ^b
E2	28.14±0.86 ^c	2.04±0.37 ^c	0.26±0.01 ^{cd}	4.37±0.51 ^b	n.d. ^b
E3	30.53±1.79 ^c	1.53±0.42 ^c	0.73±0.02 ^c	5.41±0.11 ^{ab}	n.d. ^b
MP1	34.69±0.74 ^b	1.30±0.28 ^c	n.d. ^d	6.00±0.02 ^{ab}	n.d. ^b
MP2	27.99±1.05 ^c	1.91±0.12 ^c	n.d. ^d	5.46±0.21 ^{ab}	n.d. ^b
MP3	29.83±0.38 ^c	0.69±0.05 ^c	n.d. ^d	6.11±0.26 ^{ab}	n.d. ^b
RF1	30.74±1.73 ^c	2.38±0.56 ^c	n.d. ^d	6.93±1.29 ^a	n.d. ^b
RF2	30.63±2.00 ^c	2.12±0.05 ^c	n.d. ^d	5.72±0.02 ^{ab}	n.d. ^b
RF3	30.70±1.13 ^c	1.06±0.06 ^c	n.d. ^d	6.89±0.31 ^a	n.d. ^b

These results pointed out that, besides the soluble β -D-glucans, other interesting carbohydrates and eritadenine can be extracted with boiling water from Shiitake mushrooms. However, the insoluble fractions could also be utilized as a source of hypocholesterolemic compounds such as insoluble β -D-glucans, chitins and ergosterol.

Conclusions

Maceration of shiitake powder in water at 98 °C for 1 h at pilot scale (30 L) led to the production of up to 80 L of β -D-glucan-rich extracts with concentrations of 1.9-4.2% total soluble substances (TSS). Higher or longer heat exposure of the substrate (> 2 h at 98 °C and only 20 min at 120 °C) had negative effect on β -D-glucan content. The optimal substrate to solvent ratio was established as 50 g/L, being higher ratios detrimental for the following microfiltration process.

More than 80 L of Shiitake extracts with up to 2.9% TSS and up to 1400 NTU turbidity could be successfully filtered by the studied CeraMem microfiltration membrane (0.5 μ m pore size and 0.1 m² filtration surface). The treatment produced an outstanding extract clarification, reducing turbidity to less than 0.5 NTU. Filtration fluxes of 70-60 L/(h·m²) could be easily obtained at relatively low transmembrane pressure (0.7 bar) and temperature (20 °C). Worth to mention was the fact that membrane permeability was completely recovered after filtration of so heavy suspensions. Nevertheless, it should be indicated that extracts with higher suspended particle loads might be detrimental for the membrane performance and should be avoided. Cold-settling contributed positively to the successful extracts clarification.

Large volumes (> 70 L) of clarified extracts with 2.2-3.1% TSS could be efficiently concentrated to 6-7 L by reverse osmosis or nanofiltration, corresponding to concentration factors of 8 to 10. Filtration fluxes greatly depend of the content of extract TSS and their decrease was faster when the initial content of the extract TSS was higher. Values of 80 to 20 L/(h·m²) were usual at transmembrane pressure of 25-29 bar and temperature of 20 °C. The Nanomax50 NF membrane gave higher filtration flux.

Independently of the variations tested during the extraction and filtration treatments, extracts with 13.8-16.9% (dry weight) β -D-glucans were obtained. However, effective enrichment of β -D-glucans was achieved only in the treatment carried out with the Nanomax50 NF membrane, due to its higher capacity to eliminate electrolytes. Apart from β -D-glucans, the obtained extracts contained also other hypocholesterolemic molecules such as eritadenine at concentration of up to 7 mg/g.

Nevertheless, it must be emphasized that 36-41% of water insoluble β -D-glucans and other interesting compounds such as ergosterol and chitins (respectively 0.5 and 9% (w/w)), remained in the insoluble fraction, suggesting that this fraction might be an important source of these compounds.

Acknowledgments

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Manuscript 4

Isolation and comparison of α - and β -D-glucans from shiitake mushrooms (*Lentinula edodes*) with different biological activities

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Abstract

A polysaccharide-enriched extract obtained from *Lentinula edodes* was submitted to several purification steps to separate three different D-glucans with β -(1 \rightarrow 6), β -(1 \rightarrow 3),(1 \rightarrow 6) and α -(1 \rightarrow 3) linkages, being characterized through GC-MS, FT-IR, NMR, SEC and colorimetric/fluorimetric determinations. Moreover, *in vitro* hypocholesterolemic, antitumoral, anti-inflammatory and antioxidant activities were also tested. Isolated glucans exerted HMGCR inhibitory activity, but only β -(1 \rightarrow 6) and β -(1 \rightarrow 3),(1 \rightarrow 6) fractions showed DPPH scavenging capacity. Glucans were also able to lower IL-1 β and IL-6 secretion by LPS-activated THP-1/M cells and showed cytotoxic effect on a breast cancer cell line that was not observed on normal breast cells. These *in vitro* results pointed important directions for further *in vivo* studies, showing different effects of each chemical structure of the isolated glucans from shiitake mushrooms.

Introduction

Mushroom D-glucans showed interesting industrial applications in agronomic, food, cosmetic and therapeutic areas. Such glucans might present different branching degrees, molecular mass and solubility [1, 2]. Therefore, the correlations between their chemical structures and their biological properties were deeply studied. According to their anomericity, it is possible to encounter α -D-glucans and β -D-glucans in mushroom fruiting bodies, although mixed α/β -D-glucans were also described [3]. The anomericity associated with different linkages may drastically influence tridimensional configuration and solubility; consequently, it might also modulate glucan bioactivities [4-6].

The most commonly isolated glucans from fungi are β -D-glucans, and a large variety of beneficial effects on human health was described for them, such as immunomodulatory, antitumoral, hypolipidemic or antimicrobial activities [6]. However, α -D-glucans and mixed α/β -D-glucans were less frequently isolated although they both showed antioxidant activities [7]. Furthermore, α -D-glucans were also described as compounds with interesting immunomodulatory, antitumoral, hypoglycemic and hypolipidemic properties [8-10].

Shiitake (*Lentinula edodes*) is the most popular edible mushroom in global market [11], highly valued in oriental and recently in occidental cuisine because of their characteristic flavor. This mushroom includes molecules inducing positive effects on human health such as phenolic compounds and ergothioneine (antioxidant activity), ergosterol, β -D-glucans, and eritadenine (hypocholesterolemic properties), antihypertensive peptides, lenthionine (with antithrombotic capacity), among others (see Introduction section), however, lentinan deserves special attention. It is a well-characterized glucan consisting of a main chain of (1 \rightarrow 3)-linked β -D-glucopyranose units, substituted at O-6 by β -D-glucopyranose, at a frequency of two branches for every five units from the main chain. This polysaccharide attracted clinical interest because of its strong *in vitro* and *in vivo* antitumor action as well as immunomodulatory and antiviral capacities [12]. Moreover, certain α -D-glucans such as an (1 \rightarrow 3)- α -D-glucan and glycogen ((1 \rightarrow 4),(1 \rightarrow 6)- α -D-glucan) were also detected

in MAE (microwave-assisted extraction) and hot water extractions (Chapter 3, Manuscript 1 and 2), although their potential bioactivities are nowadays not so well studied as lentinan.

Several purification procedures were developed to separate these molecules and to test their individual bioactivities. Freeze-thawing separation, treatment with solvents, dialysis, ultrafiltration and column fractionation were usually utilized since they are simple methods [13], however, polysaccharides frequently form intermolecular interactions yielding complex polymers difficult to isolate. Mushroom glucans also showed this tendency but a recent study indicated a simple and effective procedure to solve this issue and separate different glucan structures [2].

In this work, a crude polysaccharide fraction obtained from shiitake mushrooms was submitted to the novel procedure and three different glucans were isolated: a branched (1→3),(1→6)- β -D-glucan, a linear (1→3)- α -D-glucan and a mixed fraction composed mainly by a linear (1→6)- β -D-glucan with low levels of (1→3)- β -D-glucan. The chemical structures were defined by colorimetric/fluorimetric procedures, GC-MS, SEC, FT-IR and NMR. Furthermore, the antioxidant and hypocholesterolemic activities of the glucans were tested *in vitro* and their immunomodulatory effects and antitumor properties were investigated using cell cultures on THP-1 and breast tumor cell lines, respectively.

Materials and methods

Fungal material

Powdered *Lentinula edodes* S. (Berkeley) fruiting bodies (particle size < 0.5 mm, moisture < 5%) were purchased from Glucanfeed S.L. (La Rioja, Spain) and stored in darkness at -20 °C until further use.

Reagents

Absolute ethanol was obtained from Panreac and sodium borohydride (NaBH_4), sodium hydroxide pellets, glycine, D-glucose, glucosamine hydrochloride, aniline blue diammonium salt 95%, trifluoroacetic acid, pyridine, acetic anhydride,

copper(II) sulfate (CuSO_4), deuterated dimethylsulfoxide ($\text{Me}_2\text{SO}-d_6$), Congo Red, citric acid, dextran (M_w 35,000-45,000 g/mol), RPMI 1640 medium and phorbol 12-myristate 13-acetate (PMA), DPPH (2,2-diphenyl-1-picrylhydrazyl), DMEM medium, dimethyl-sulfoxide, ascorbic acid, horse serum, fetal bovine serum, hydrocortisone, recombinant EGF and insulin were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA).

Extraction and purification of polysaccharides

Shiitake powder was submitted to hot water extraction (98 °C, 1 h) as described in Chapter 3, Manuscript 3 and the soluble fraction was previously studied. The insoluble fraction containing high levels of glucans (40% β -D-glucans dry weight) was utilized to carry out the purification procedures (Figure 1).

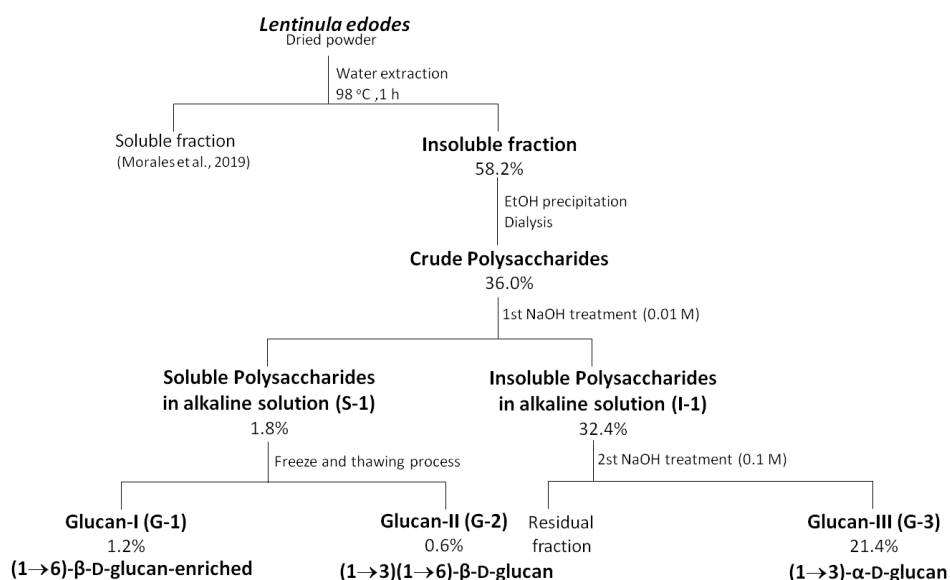


Figure 1. Scheme of extraction and purification of glucans obtained from shiitake powder. Indicated yield (%) was calculated on basis of the initial dry weight of shiitake mushroom powder.

Ethanol precipitation was performed by adding 3 volumes of ethanol, mixing vigorously and keeping the mixture overnight at 4 °C. The precipitated polysaccharides were recovered after centrifugation (10000 rpm, 15 min) and the

pellets were suspended in water and dialysed (2 KDa M_r cut-off membrane) against water for 24 h. The crude polysaccharides were freeze-dried and submitted to the first alkaline treatment (stirring with 0.01 M NaOH solution at 22 °C, for 1 h). After this period, the solution was cooled down to 4 °C and then centrifuged (8000 rpm, 10 °C, 20 min). Soluble (S-1) and insoluble (I-1) polysaccharides resulting from the alkaline treatment were neutralized with acetic acid and dialyzed (2 KDa M_r cut-off membrane) against water for 24 h and then freeze-dried. S-1 was submitted to a freeze-thawing process [14], and subdivided into two new fractions based on their solubility in water: Glucan-I (G-1) and Glucan-II (G-2). Due to high insolubility, fraction I-1 was submitted to a second and stronger alkaline treatment (stirring with 0.1 M NaOH solution; at 22 °C, for 1 h) [2], yielding two new fractions, although only the insoluble one (named Glucan-III, G-3) was used in this study. Extraction yields were calculated based on the initial dry weight of shiitake mushroom powder.

GC-MS analysis

The monosaccharide composition of the fractions (G-1, G-2, and G-3) was determined by hydrolyzing the samples (1 mg) with 2M trifluoroacetic acid at 100 °C for 8 h followed by evaporation to dryness. The dried samples were dissolved in distilled water (100 μ L) and NaBH₄ (1 mg) was added. Then, solution was kept at room temperature overnight to reduce aldose into alditols [15] and later, the samples were dried and the NaBH₄ excess was neutralized by adding acetic acid and then removed with methanol (twice) under a compressed air stream. Alditols acetylation was performed in pyridine-acetic anhydride (200 μ L; 1:1 v/v) for 30 min at 100 °C. Pyridine was removed by washing with 5% CuSO₄ solution and the resulting alditol acetates were extracted with chloroform. The samples were injected into an SH-Rtx-5ms (30 m x 0.25 mm ID x 0.25 μ m thickness phase). The column was connected to a GC-2010 Plus gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a Combipal autosampler (AOC 5000) and coupled to a triple quadrupole mass spectrometer TQ 8040. The injector and ion source were held at 250 °C and helium at 1 mL/min was used as carrier gas. The oven temperature was programmed from 100 to 280 °C at 10 °C/min with a total analysis time of 30 min. The samples were prepared in hexane with 1 μ L being injected with a split ratio of 1:10. The mass

spectrometer was operated in the full-scan mode over a mass range of m/z 50-500 before selective ion monitoring mode, both with electron ionization at 70 eV. Selective ion monitoring mode was used for quantification and *GCMS solution* software (Tokyo, Japan) was used for data analysis. The obtained monosaccharides were identified by their typical retention time compared to commercial available standards. Results were expressed as mol%, calculated according to Pettolino et al. (2012) [16].

NMR spectroscopy

NMR spectra (^1H , ^{13}C and HSQC-DEPT) from the different fractions were obtained using a 400 MHz Bruker model Advance III spectrometer with a 5 mm inverse probe, and the analyses were performed at 70 °C. The samples (30 mg) were dissolved in $\text{Me}_2\text{SO}-d_6$ and were centrifuged (10000 rpm, 22 °C, 2 min) to remove insoluble material, therefore only the soluble fractions of G-1, G-2 and G-3 were analyzed. Chemical shifts are expressed in ppm (δ) relative to $\text{Me}_2\text{SO}-d_6$ at 39.7 (^{13}C) and 2.40 (^1H).

FT-IR and SEC analyses

Infrared analysis was performed in a Vertex 70 spectrometer (Bruker, Germany) with attenuated total reflectance (ATR). Aliquots of the dried samples G-1, G-2, and G-3 were prepared using KBr disc technique and directly submitted to infrared analysis with 32 scans from 410 to 4000 cm^{-1} with resolution of 4 cm^{-1} .

SEC analysis was performed at 40 °C using as mobile phase NaNO_3 0.1 mol/L containing sodium azide 200 ppm under a flow rate of 0.4 mL/min in a Viscotek-SEC multidetector-system. This system was equipped with an OH-Pack Shodex SB-806M HQ column (size exclusion limits of 2×10^7 g/mol) coupled to laser light scattering detector model 270 dual detector with low angle 7° (LALLS) and right angle 90° (RALLS) with λ at 632.8 nm and to a RI (Viscotec VE3580) detector. Aliquots of samples were dissolved in the eluent (1 mg/mL) and then filtered through 0.22 μm cellulose membrane prior to injection. Results were analyzed with OmniSEC software (Malvern Co., USA) and Mw was calculated only for soluble samples.

Colorimetric determinations with Congo red

Determination of triple helix conformation was performed according to Smiderle et al. (2014) [17]. Congo red was dissolved (80 μ M) in 50 mM NaOH solution. Dextran (1 mg/mL) was used as random coil control and Congo red alone was considered as negative control. Studied samples (G-1, G-2, G-3) were added (1 mg/mL) to Congo red solutions and spectra were recorded on an Evolution 600 UV-vis spectrophotometer (ThermoFisher Scientific, Spain) in intervals of 10 nm from 400 to 640 nm.

Fluorimetric determinations

The determination of (1 \rightarrow 3)- β -D-glucans was carried out as described in Chapter 3, Manuscript 1. Briefly, purified samples (G-1, G-2, G-3) were solubilized (2.5-100 μ g/mL) in 300 μ L of 0.05 M NaOH with 1% NaBH₄ in 2 mL reaction tubes. After that, 30 μ L of 6 M NaOH and 630 μ L of dye mix (0.1% aniline blue: 1M HCl: 1M glycine / NaOH buffer pH 9.5; 33:18:49) was added and the mixed samples were incubated at 50 °C for 30 min in a water bath and transferred to a 96-well plate to carry out fluorimetric analysis (excitation: 398 nm; emission: 502 nm) in a M200 Plate Reader (Tecan, Mannedorf, Switzerland).

Determination of HMGCR inhibitory activity

Purified samples were solubilized in water (G-1) or water/DMSO (G-2, G-3, 1:0.063, 10 mg/mL) and applied (20 μ L) into a 96-wells plate. Their inhibitory activity was measured using the commercial HMGCR (3-hydroxy-3-methylglutaryl coenzyme A reductase) activity assay (Sigma-Aldrich, Madrid, Spain) according to the manufacturer's instructions by monitoring their absorbance change (340 nm) at 37 °C using a 96-wells microplate reader BioTek Sinergy HT (BioTek, Winooski, USA). Pravastatin was used as a control for positive inhibition.

Determination of free radical scavenging activity

The scavenging activity of the isolated glucans against the stable free radical DPPH• (2,2-diphenyl-1-picrylhydrazyl) was determined, using different concentrations of the fractions G-1, G-2 and G-3 (1000; 300; 100; 30; 10; 3; and 1 µg/mL). This method was adapted from Kanazawa et al. (2016) [18]. Briefly, the tested fractions were, separately, mixed with DPPH methanol solution (40 µg/mL), and absorbance was immediately read at 517 nm in an Epoch Microplate Spectrophotometer. Ascorbic acid (50 µg/mL) and PBS (or PBS/DMSO, 1:0.063, for G-2 and G-3) were used as positive and negative controls, respectively. The blank of each sample/control was read at 517 nm before the addition of DPPH solution. A standard curve of DPPH (ranging from 0 to 60 µM of DPPH) was read at the same wavelength to calculate its concentration relative to absorbance.

Macrophage cultures and immunomodulatory testing

The human monocyte THP-1 cell line was obtained from ATCC and cultured with supplemented RPMI 1640 medium (10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin, 2mM L-glutamine and 0.05 mM β-mercaptoethanol). For differentiation into macrophages, THP-1 cells were seeded (5×10^5 cells/mL) in 24 well-plate with 100 ng/mL phorbol 12-myristate 13-acetate (PMA) and maintained for 48 h at 37 °C under 5% CO₂ in a humidified incubator.

Firstly, the glucans cytotoxicity (G-1, G-2, G-3) was evaluated in differentiated macrophages using 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide (MTT) protocol [19]. Afterwards, the macrophages were washed with PBS and then replaced with serum-free medium containing LPS (0.05 µg/mL) and subtoxic concentrations of the glucans. After 10 h of incubation, cells supernatants were collected and store at -20°C until use.

Pro-inflammatory cytokines TNF-α (Tumour necrosis factor alpha), IL-1β (Interleukin 1 beta) and IL-6 (Interleukin 6) were measured in the supernatants by BD Biosciences Human ELISA set (Aalst, Belgium) following the manufacturer's instructions. The quantification was calculated considering positive controls (cells

stimulated with LPS) as a 100% cytokine secretion. The colour generated was determined by measuring the OD at 450 nm using a multiscanner autoreader (Sunrise, Tecan). The assays were conducted in three independent experiments, in triplicated wells.

Inhibitory activity of tumoral cells growth

MDA-MB-231 breast cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (1%). The mammary non-tumorigenic epithelial cells MCF-10A was cultured in DMEM medium supplemented with 5% horse serum, 0.5 mg/mL hydrocortisone, 20 ng/mL recombinant EGF and 10 µg/mL insulin. Both cell lines were obtained from ATCC and they were maintained in a humidified atmosphere containing 5% CO₂ at 37 °C.

The normal (MCF-10A) and tumoral (MDA-MB-231) cells were seeded into 96-well plates (4×10^4 cells/mL) for 24 hours to adhere. Later on, the cells were exposed to treatment with G-1, G-2, or G-3 (at 10, 50 or 250 µg/mL), for 24 and 48 hours. The samples were solubilized in sterile PBS (G-1) or in a mixture of sterilized PBS:dimethyl sulfoxide (3:1) (G-2 and G-3) until complete solubilization. The presence of dimethyl sulfoxide at this concentration (1.25%) was not toxic for the cells (data not shown). Afterwards, the cell viability was determined by two different assays in separated plates: MTT test [19] and Live/Dead® Viability/Cytotoxicity kit (according to the manufacturer). PBS alone and the mixture of PBS:dimethyl sulfoxide (3:1) were used as control and the cell viability was expressed as a percentage of control cells. The assays were conducted in three independent experiments, in quadruplicated wells for MTT and sextuplicated wells for Live/Dead® Viability/Cytotoxicity kit. After the treatment MTT plates were read at 595 nm and Live/Dead plates were read in the InCell Analyzer 2000 Imaging System (GE, Healthcare, UK). Green and Red fluorescence intensity are recorded by the equipment from 4 fields in each well and the values of live and dead cells are calculated by the mean of each well.

Statistical analysis

Differences were evaluated at 95% confidence level ($P \leq 0.05$) using a one-way analysis of variance (ANOVA) followed by Tukey's or Bonferroni's Multiple Comparison test. Statistical analysis was performed using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA).

Results and discussion

Isolation and chemical characterization of the purified fractions

Several fractions were isolated from shiitake mushrooms (Figure 1). After hot water extraction, the insoluble material yielded a crude polysaccharide extract (58.2% dw) containing 91.4% glucose and smaller amounts of mannose and galactose. HSQC-DEPT spectrum (Figure 2a) of this fraction showed the presence of α - and β -D-glucans evidenced by signals relative to C-1/H-1 of α -D-Glcp at δ 99.2/4.98 and at δ 102.1/4.41 and 102.7/4.17 of β -D-Glcp. Signals at δ 82.6/3.56 and δ 85.7/3.38 indicated C-3 *O*-substitution, probably related to respectively α -D-Glcp and β -D-Glcp units. The (1 \rightarrow 3)-linkages are commonly found in β -D-glucans isolated from mushrooms [3] and α -(1 \rightarrow 3)-linkages for glucans, although less observed, were previously detected in *Lentinula edodes* extracts (Chapter 3, Manuscript 2) and other mushrooms such as *Fomitopsis betulina* [2].

Based on literature and considering that the crude polysaccharide fraction might be a mixture of two or more glucans, the crude extract was further treated with mild alkaline conditions [2] to remove possible inter-molecular interactions between glucans. Then, two fractions were obtained: S-1 (soluble) and I-1 (insoluble) showing different signals in their NMR spectra. The spectrum of the soluble fraction S-1 (Figure 2b) showed intense signals at δ 102.4/4.43; 102.6/4.27 and 102.8/4.17, confirming β -configuration of D-Glcp and at 85.9/3.39 ppm relative to *O*-3 substitution of β -D-Glcp units; while I-1 spectrum (Figure 2c) showed more intense signal at 99.2/5.00 ppm and 82.9/3.55 ppm indicating the presence of α -D-Glcp (1 \rightarrow 3)-linked. Small contaminations of other glucans were also noticed in both

spectra, although the intensity of the main anomeric signals and (1→3)-linked signals of each glucan (α - and β -) was an indicative of successful purification method.

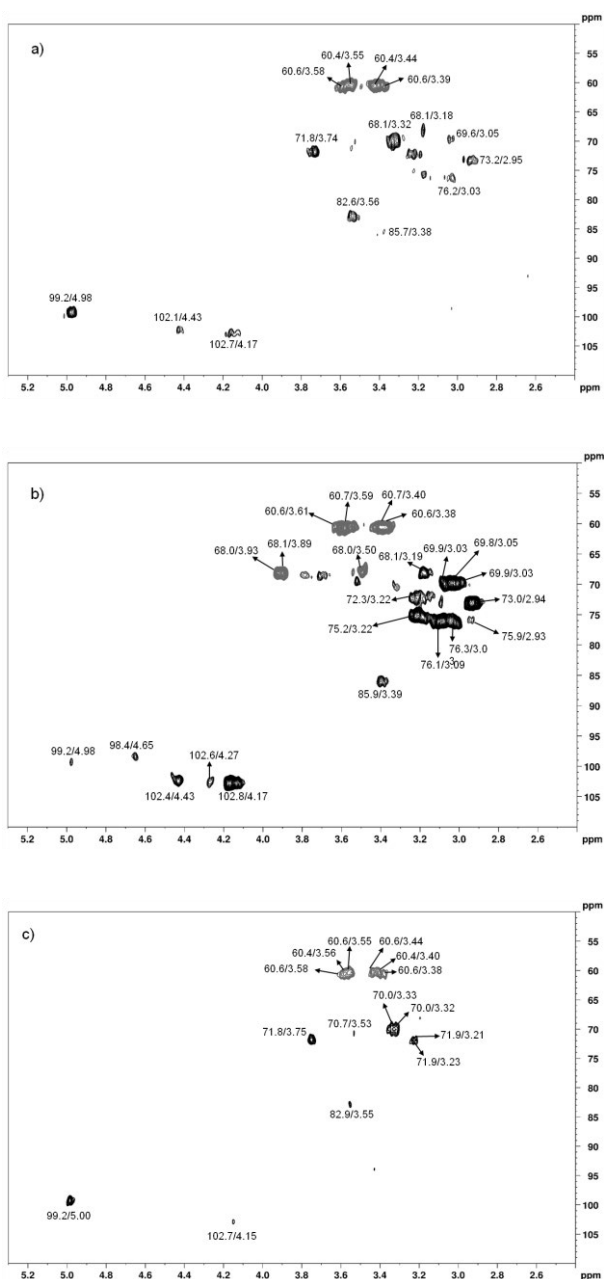


Figure 2. HSQC-DEPT NMR spectra of crude polysaccharides fraction (a), S-1 (b) and I-1 (c). Experiment was performed in Me₂SO at 70 °C (chemical shifts are expressed in δ ppm).

To refine the samples purification, fraction S-1 was submitted to freeze-thawing process and divided into two fractions according to their solubility in cold water: soluble (G-1) and non-soluble (G-2). The monosaccharide composition of G-1 and G-2 were 87.6% and 81.4% glucose, respectively, and low contents of galactose and mannose (Supplementary Figures 1 and 2). FT-IR spectra of both fractions showed characteristic bands of carbohydrates (Supplementary Figure 4). Strong broad band between 3000 cm^{-1} and 3500 cm^{-1} , centered at $\sim 3400\text{ cm}^{-1}$ indicate the presence of OH stretching vibration, and were observed in both spectra. The absorption observed at 1089 cm^{-1} (for G-1) and at 1093 cm^{-1} (for G-2) are characteristic of β -D-glucans [20, 21]. G-1 presented also an evident absorption at 1436 cm^{-1} , which is representative of CH_2 [21], and this suggests the presence of a linear glucan in G-1. A small peak was also observed in G-2 spectrum relative to CH_2 at 1456 cm^{-1} . Characteristic absorptions of protein was observed in both spectra at 1666 cm^{-1} (G-1) and 1670 cm^{-1} (G-2) [20].

FT-IR data corroborate the NMR results, the HSQC-DEPT of G-1 (Figure 3a) suggested the major presence of a linear $(1\rightarrow 6)$ - β -D-glucan that was not previously reported in shiitake, but was detected in other species, such as *Agaricus* spp. [22]. The signals corresponding to C-1/H-1 were observed at δ 102.7/4.26 and the inverted signals at δ 69.0/3.95 and 69.0/3.59, indicated the O-6 substitution, confirming the presence of a $(1\rightarrow 6)$ - β -D-glucan. Other four signals were evidenced corresponding to C-2/H-2 (δ 73.0/3.09), C-3/H-3 (δ 75.7/3.26), C4-H-4 (δ 69.6/3.20) and C5-H-5 (δ 74.7/3.37) of the main chain. However, the signals at 60.8/3.65 and 60.8/3.50 ppm indicated the presence of another polysaccharide with non-substituted CH_2 that could be traces of the $(1\rightarrow 3)$ - β -D-glucan observed in the other fractions. On the other hand, HSQC-DEPT of G-2 fraction (Figure 3b) showed typical signals of a $(1\rightarrow 3)$ - β -D-glucan, branched at O-6 by β -D-Glcp units, commonly found in shiitake and other mushrooms [13]. The intense signals at δ 102.6/4.46 and 102.8/4.17 were relative to C1/H1, at δ 85.9/3.39 indicated C3/H3 O-substituted, and at δ 68.1/3.93 and 68.1/3.50, confirmed CH_2 -O-substituted of β -D-Glcp units. All the assignments were confirmed with literature data [23, 24]. G-1 and G-2 presented a mass-average molar mass (M_w) of 6,536 g/mol and 14,272 g/mol, respectively. M_w was calculated

using $\partial n/\partial c$ value of 0.133 mL/g [25] and the recovery from SEC column was 100% for (1→6)- β -D-glucan; while for (1→3)-(1→6)- β -D-glucan, $\partial n/\partial c$ value was 0.157 mL/g [24] and the recovery from SEC column was 70 %.

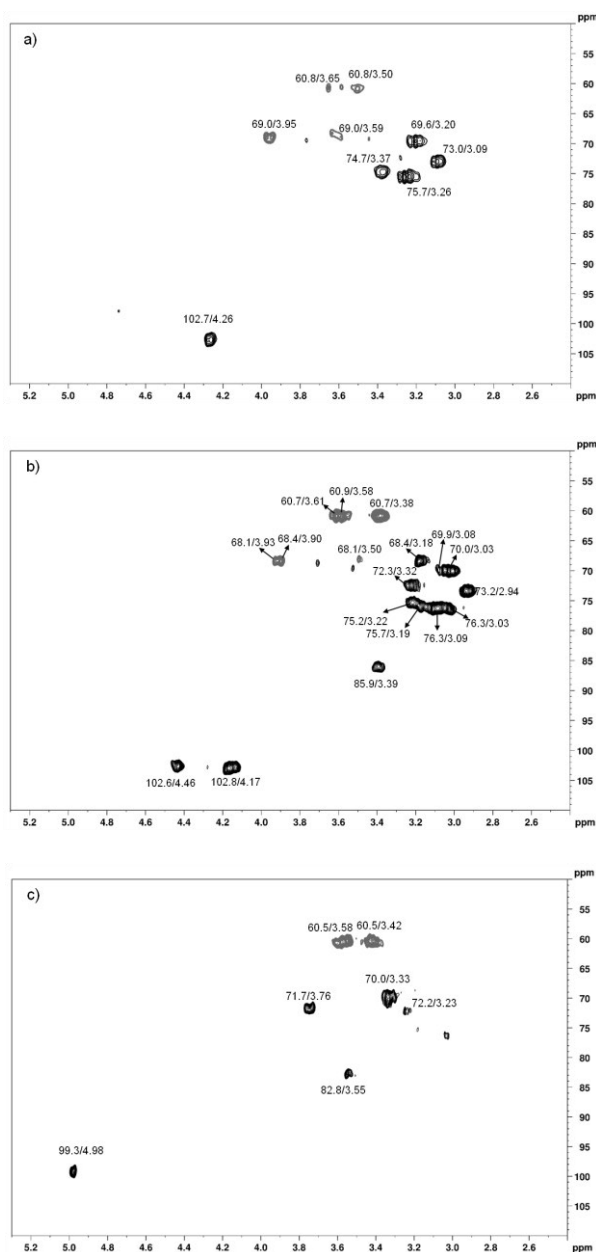


Figure 3. HSQC-DEPT NMR spectra of G-1 (a); G-2 (b) and G-3 (c) fractions. Experiments were performed in Me_2SO at 70 °C (chemical shifts are expressed in δ ppm).

Finally, when the insoluble fraction (I-1) was submitted to a second and stronger alkaline treatment, a residual fraction (not studied) and a highly insoluble fraction G-3 were obtained. The latter fraction included 100% glucose in its composition according to the GC-MS analysis (Supplementary Figure 3). FT-IR spectrum of G-3 (Supplementary Figure 4) presented similar absorption bands of the other two glucans, such as OH stretching vibration characteristic peaks at 3471 cm^{-1} , CH_2 absorption at 1463 cm^{-1} , however this sample did not show the typical band at $\sim 1080\text{ cm}^{-1}$ (relative to β -glucan). Instead, it was observed vibration ranging from $597 - 729\text{ cm}^{-1}$, which indicates α -linkages [20, 21]. Characteristic absorption of proteins was also observed for this sample at 1668 cm^{-1} .

More information about the chemical structure of G-3 glucan was obtained on its NMR spectrum (Figure 3c), that showed main signals at 99.3/4.98 (C-1/H-1), 72.2/3.23 (C-2/H-2), 82.8/3.55 (C-3/H-3), 70.0/3.33 (C-4/H-4), 71.7/3.76 (C-5/H-5), inverted 60.5/3.58 and 60./3.42 (C-6/H-6; CH_2) ppm, confirming that the purification process was efficient to isolate the $(1\rightarrow3)\text{-}\alpha\text{-D-glucan}$ as performed previously by de Jesus et al. (2018) [2]. This fraction was also injected in SEC column, although, due to its high insolubility, the recovery from the column was 18%, and therefore the M_w value was not possible to be estimated.

Colorimetric determination with Congo red was used to determine the presence of triple helix conformation since Ogawa et al. (1972) [26] stated that polysaccharides with this tridimensional structure could form complex with Congo red, leading to a bathochromic shift of the maximum visible absorption (490 nm) of the Congo red spectrum. Dextran was used as random coil control and showed similar behaviour than Congo red solution, with no bathochromic shift. Fractions that contained the $(1\rightarrow6)\text{-}\beta\text{-D-glucan}$ (G-1) and $(1\rightarrow3)\text{-(}1\rightarrow6\text{)-}\beta\text{-D-glucan}$ (G-2) displayed a bathochromic shift of 10 nm (Figure 4a), suggesting triple helix conformations for such polysaccharides. On the other hand, fraction G-3 (Figure 4a), which contained the $(1\rightarrow3)\text{-}\alpha\text{-D-glucan}$, showed no bathochromic shift, indicating random coil conformation such as the control of dextran. This bathochromic shift was also observed for a linear $(1\rightarrow3)\text{-}\beta\text{-D-glucan}$ isolated from *Cordyceps militaris* [17] and a branched $(1\rightarrow3)\text{-(}1\rightarrow6\text{)-}\beta\text{-D-glucan}$ isolated from *Pleurotus ostreatus* [27]. An

(1 \rightarrow 4)- α -D-glucan obtained from *P. ostreatus* by the latter authors also presented no bathochromic shift as the (1 \rightarrow 3)- α -D-glucan isolated in this study. These results confirm that different linkage types and anomeric configurations are strictly related to the tridimensional structure and, consequently to the therapeutic application of the glucans [4].

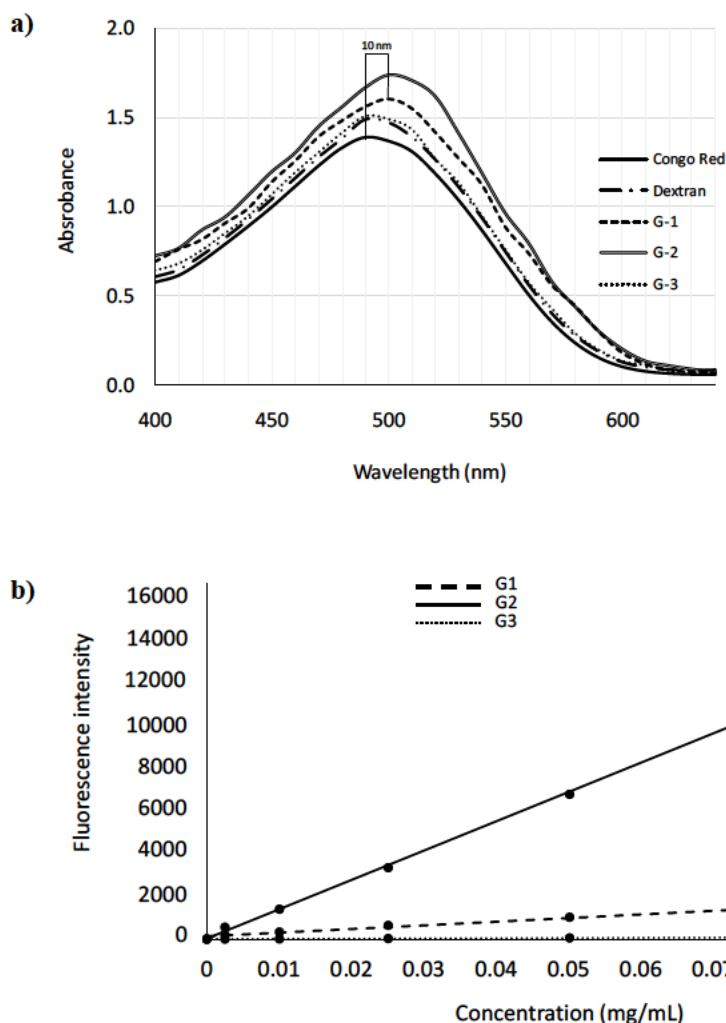


Figure 4. a) Absorption spectra of Congo red (control) and Congo red with dextran, G-1, G-2 and G-3 and b) fluorescence intensity of G-1, G-2 and G-3 at different concentrations.

Aniline blue/sirofluor is a fluorophore described and widely utilized for its specificity or preference to bind to (1→3)- β -D-glucans (Chapter 3, Manuscript 1) [28]. The branched β -D-glucan (G-2) exhibited intense fluorescence when compared to the linear β -D-glucan (G-1) and the linear α -D-glucan (G-3). The G-1 glucan showed slight fluorescence, while G-3 showed no fluorescence (Figure 4b). Therefore, these results were in concordance with the NMR indications. It was possible to observe that the branched β -D-glucan (G-2) showed the highest fluorescence as also observed in Chapter 3, Manuscript 1. However, the fraction G-1, including mainly a (1→6)- β -D-glucan, showed a slight fluorescence, differing from results observed that detected no fluorescence for linear (1→6)- β -D-glucans (Chapter 3, Manuscript 1). This might indicate that the fraction G-1 still contained a small amount of the branched (1→3)-(1→6)- β -D-glucan. Furthermore, the fluorescence absence of G-3 fraction confirmed that the (1→3)- α -D-glucan fraction excluded the presence of β -D-glucans.

HMGCR inhibitory activity

β -D-Glucans were pointed as hypocholesterolemic polysaccharides since they reduced cholesterol and bile acids concentrations in the intestinal lumen impairing their absorption by enterocytes. The precise mechanism is not completely elucidated but they might increase intestinal viscosity or/and scavenge small compounds within their complex structures leading to lower plasma cholesterol levels [29]. Moreover, Gil-Ramirez et al. (2017) [30] found that certain mushroom β -D-glucans such as curdlan or schizophyllan were able to inhibit (*in vitro*) the activity of the 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR), the key enzyme in the biosynthesis of endogenous cholesterol and target of drugs such as statins. Tong et al. (2015) [31] also observed reduction of HMGCR activity in hamsters liver when administrated barley β -D-glucans. Thus, the HMGCR inhibitory activity of the isolated glucans was evaluated and results indicated that they showed remarkable inhibitory activities (Table 1).

Table 1. HMGCR inhibitory activity (%) of G1, G2 and G3. (a-c) Different letters denote significant differences ($P < 0.05$) between samples.

Sample	HMGCR inhibition (%)
G-1	82.63±0.76 ^b
G-2	74.57±0.29 ^c
G-3	89.26±0.83 ^a

The G-2 fraction containing the (1→3),(1→6)-β-D-glucan reduced the enzyme activity up to similar levels than reported for schizophyllan, a polysaccharide with similar structure [30]. The linear β-D-glucan (G-1) and particularly the (1→3)-α-D-glucan (G-3) showed even higher inhibition capacities, higher than other α-D-glucans such as dextran [30].

DPPH scavenging capacity

The antioxidant activities of glucans and other polysaccharides are frequently related to some of their therapeutic benefits [7, 10]. Therefore, the free radical scavenging activities of the fractions G-1, G-2, and G-3 were also investigated using DPPH• as radical. Only glucan fractions G-1 and G-2 showed scavenging effect on the DPPH radical, being G-1 the fraction with higher antioxidant activity (Figure 5) with an IC₅₀ of 183.8 µg/mL. This linear (1→6)-β-D-glucan showed higher chelating index than other fungal polysaccharides such as a heteropolysaccharide from *Pleurotus ostreatus*, which exhibited an IC₅₀ of 1.43 mg/mL [32]; and a glucan-rich heteropolysaccharide from *Inonotus obliquus*, with an IC₅₀ of 1.3 mg/mL [33]. Other authors evaluated the scavenging ability of glucan-rich extracts from *Agaricus bisporus*, *Pleurotus ostreatus*, and *Coprinus attrimentarius* and observed lower scavenging activity (IC₅₀: ~5 mg/mL) for the three extracts in comparison to the linear (1→6)-β-D-glucan [34]. No other isolated glucan was evaluated on DPPH assay.

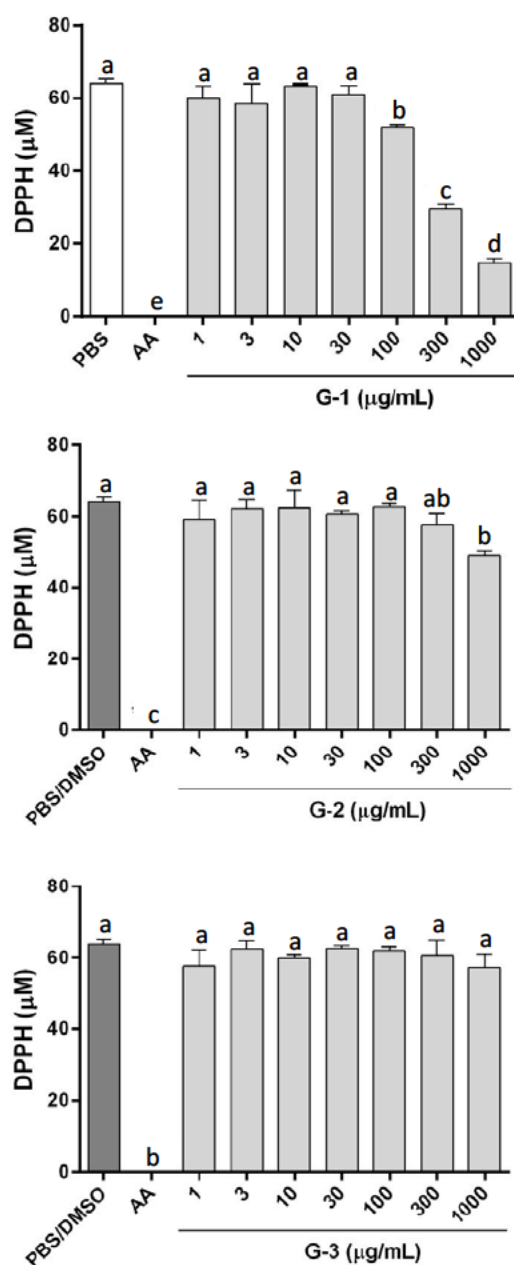


Figure 5. Effects of glucans as DPPH radical scavengers. PBS or PBS/DMSO: negative control (vehicle). AA: positive control (ascorbic acid). Different letters (a-e) denote significant differences ($P < 0.05$) between samples.

Anti-inflammatory activity on immune cells

The immunomodulatory activity of the purified glucans was also tested as their capacity to reduce the secretion of pro-inflammatory cytokines in macrophages differentiated from THP-1 human monocytes cell line. The preliminary experiments to assess the glucans cytotoxicity indicated that when applied up to 10 µg/mL, the viability of THP-1 macrophages was not affected (data not shown). Thus, the immunomodulatory activity was tested in this subtoxic concentration. The THP-1 macrophages stimulated with LPS (positive control) exhibited a significant release of the three pro-inflammatory cytokines studied (TNF- α , IL-1 β and IL-6) compared to non-stimulated cells (negative control) (Figure 6). Addition of the glucans plus LPS did not reduce the amount of TNF- α liberated in the media, but significantly decreased IL-1 β and IL-6 levels. Moreover, G-3 modulated IL-1 β secretion reaching significantly lower values when compared to G-2 (43 and 26 %, respectively), but all three glucans inhibited more than 42 % the secretion of IL-6.

Previous reports testing mushroom glucans also showed anti-inflammatory effects. For instance, (1 \rightarrow 6)- β -D-glucans from *Agaricus bisporus* and *Agaricus brasiliensis* were able to inhibit IL-1 β and COX-2 expression when administered to LPS-activated THP-1 macrophages [22]. Furthermore, a linear (1 \rightarrow 3)- β -D-glucan isolated from *Cordyceps militaris* also inhibited the expression of IL-1 β , TNF- α and COX-2 of THP-1 cells stimulated with LPS [17]. Another linear (1 \rightarrow 6)- β -D-glucan from *Pleurotus citrinopileatus* lowered the secreted levels of IL-6 and TNF- α differentiating macrophages stimulated with IFN- γ /LPS [35]. Comparing literature data with observed results, it seems that linear glucans, such as (1 \rightarrow 6)- β -D-glucan (G-1) and (1 \rightarrow 3)- α -D-glucan (G-3) produce more marked anti-inflammatory effects than the branched (1 \rightarrow 6),(1 \rightarrow 3)- β -D-glucans (G-2).

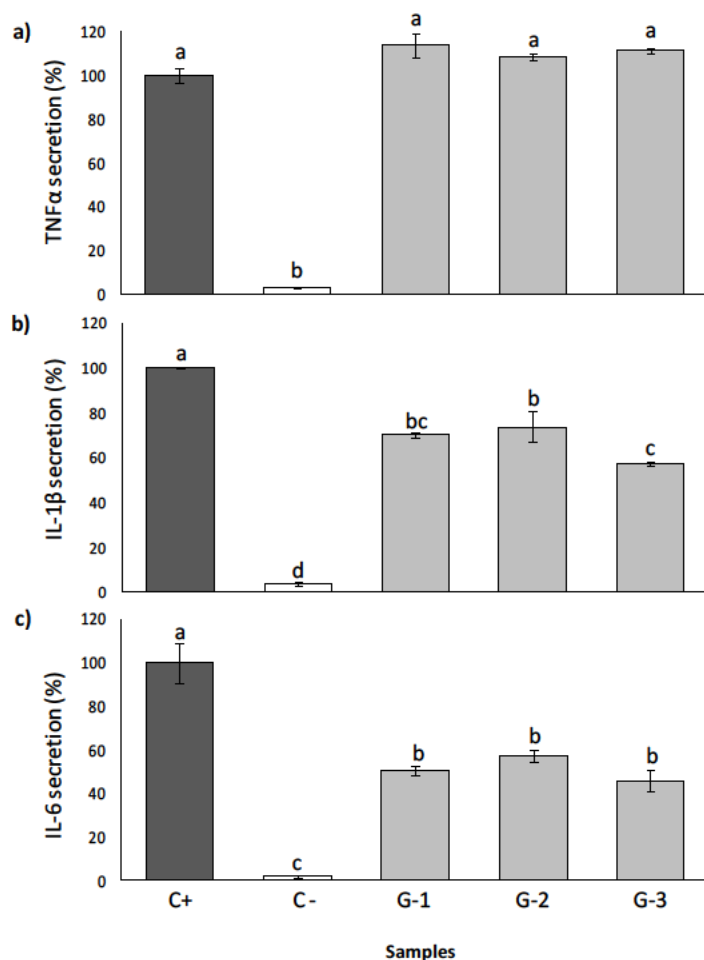


Figure 6. Levels of a) TNF- α , b) IL-1 β and c) IL-6 secreted by THP-1/M activated with LPS in presence of G-1, G-2 and G-3 (10 μ g/mL). Positive control (C+): cells stimulated with LPS but in absence of extract. Negative control (C-): non LPS-activated cells. Different letters (a-d) denote significant differences ($P < 0.05$) between samples.

Cytotoxic effect on tumor cells

The antitumor activities of mushroom glucans are usually indirectly due to the stimulation of immune system that diminishes tumor resistance [4, 8].

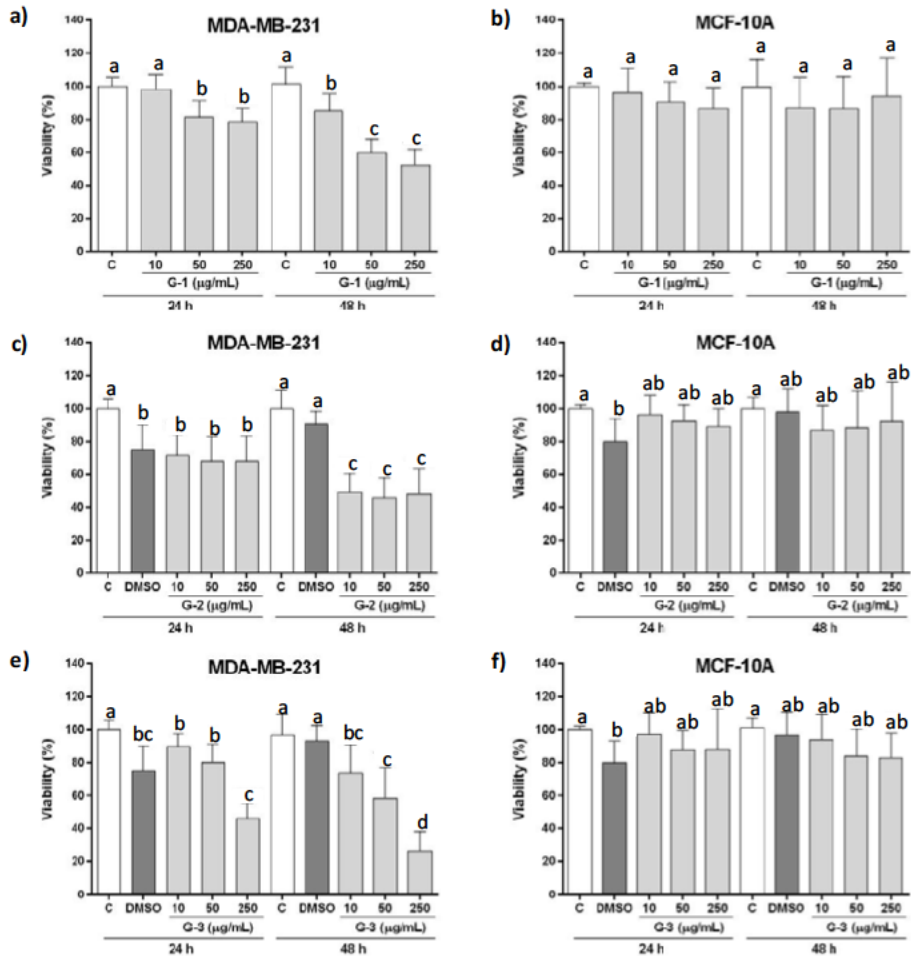


Figure 7. Cell viability of MDA-MB-231 (tumor cell line, left) and MCF-10A (normal cell line, right) measured by MTT assay, after incubation with G-1 (a, b), G-2 (c, d) or G-3 (e, f) for 24 h and 48 h. C: medium plus PBS (vehicle); DMSO: medium plus dimethyl sulfoxide (1.25%). Different letters (a-c) denote significant differences ($P < 0.05$) between samples.

However, the three isolated polysaccharides showed a direct effect on the viability of MDA-MB-231 breast tumor cells, as seen by MTT results (Figure 7a, c

and e). When the tumoral cells were separately treated with all fractions (G-1, G-2, G-3) a cytotoxic activity was noticed that was significant when applied mainly at 50 and 250 $\mu\text{g/mL}$, for 24 h and 48 h. However, when G-1, G-2 and G-3 were incubated with normal tissue breast cells (MCF-10A) no cytotoxic effect was observed (Figure 7b, d and f).

The fraction G-1 containing mainly the linear (1 \rightarrow 6)- β -D-glucan decreased the viability of tumor cell concomitant with the increase of applied concentration up to approx. 50% after 48 h of incubation when applied at 250 $\mu\text{g/mL}$. However, the branched (1 \rightarrow 6),(1 \rightarrow 3)- β -D-glucan (G-2) also diminished the viability approx. 50% (after 48 h) independently of the tested concentration (10; 50; 250 $\mu\text{g/mL}$). The highest cytotoxic activity was observed for the linear (1 \rightarrow 3)- α -D-glucan (G-3) where the noticed reduction was dependent of the concentration utilized. This glucan was able to reduce approx. 54% and 73% MDA-MB-231 cells viability after 24 h and 48 h of incubation, respectively, when applied at 250 $\mu\text{g/mL}$, being completely innocuous for MCF-10A cells.

Table 2. Cell viability of MDA-MB-231 and MCF-10A measured by Live/Dead® Viability/Citotoxicity kit, after incubation with G-1, G-2, or G-3 for 24 h and 48 h. Vehicle control: medium plus dimethyl sulfoxide (1.25%). Different letters (a-b) denote significant differences ($P < 0.05$) between samples for the same treatment time and cell line.

Cell Line: MDA-MB- 231	24h Treatment ($\mu\text{g/mL}$)				48h Treatment ($\mu\text{g/mL}$)			
	10	50	250	Vehicle control	10	50	250	Vehicle control
G1	98.47 \pm 1.32 a	99.27 \pm 0.15 a	99.04 \pm 0.22 a		95.28 \pm 3.33 b	98.62 \pm 2.12 ab	99.36 \pm 0.21 a	
G2	98.91 \pm 0.22 a	95.78 \pm 0.66 b	94.04 \pm 1.40 b	99.30 \pm 0.06 a	98.83 \pm 1.00 ab	98.04 \pm 0.18 ab	98.53 \pm 0.45 ab	99.48 \pm 0.11 a
G3	99.19 \pm 0.26 a	98.46 \pm 0.24 a	93.17 \pm 0.45 b		99.28 \pm 0.06 ab	99.19 \pm 0.07 ab	92.90 \pm 1.71 b	
MCF-10A	24h Treatment ($\mu\text{g/mL}$)				48h Treatment ($\mu\text{g/mL}$)			
	10	50	250	Vehicle control	10	50	250	Vehicle control
G1	96.85 \pm 0.15 b	97.15 \pm 0.16 b	99.25 \pm 0.10 a		97.34 \pm 0.30 b	97.78 \pm 0.34 b	99.42 \pm 0.12 a	
G2	97.40 \pm 0.20 b	99.12 \pm 0.34 a	99.93 \pm 0.04 a	97.73 \pm 0.53 b	97.73 \pm 0.31 b	98.94 \pm 0.42 a	99.89 \pm 0.02 a	97.43 \pm 0.21 b
G3	96.85 \pm 0.19 b	96.60 \pm 0.28 b	97.89 \pm 0.95 b		97.44 \pm 0.57 b	97.17 \pm 0.27 b	98.93 \pm 0.58 a	

When the cells treated, separately, with G-1, G-2 and G-3 were evaluated using Live/Dead Viability kit, which shows live cells with green fluorescence and dead cells with red fluorescence, the values of cytotoxicity were less prominent than the results observed for MTT assay, however, the statistical analysis still showed significant toxicity to MDA-MB-231 cells treated with G-2 and G-3, in comparison with the cells treated only with vehicle (Table 2).

According to the results observed, the samples G-2 and G-3 were cytotoxic for the tumor cells (MDA-MD-231) after 24 h of treatment, at 250 $\mu\text{g/mL}$ and G-3 exhibited the same effect after 48 h of treatment. By this technique, sample G-1 did not show any toxic effect to such cells. The non-tumorigenic cell line MCF-10A was not affected by the treatments and moreover it exhibited slight proliferation at the highest concentrations of the three glucans. Representative pictures can be observed on Supplementary Figures 5-11.

Conclusions

The polysaccharide-enriched extract from *L. edodes* was a valuable source of bioactive glucans that were successfully isolated using easy and effective procedure with several steps such as alkaline extractions and freeze-thawing processes. Three glucan fractions that were separated contained mainly a linear $(1\rightarrow6)\text{-}\beta\text{-D-glucan}$ with low levels of $(1\rightarrow3)\text{-}\beta\text{-D-glucan}$ (G-1), a branched $(1\rightarrow3),(1\rightarrow6)\text{-}\beta\text{-D-glucan}$ (G-2) and a linear $(1\rightarrow3)\text{-}\alpha\text{-D-glucan}$ (G-3) according to GC-MS, FT-IR and NMR analyses. M_w of G-1 and G-2 was calculated as 6,536 g/mol ($\partial n/\partial c$: 0.133 mL/g) and 14,272 g/mol ($\partial n/\partial c$: 0.157 mL/g), respectively. Congo Red colorimetric assay indicated tridimensional conformation for G-1 and G-2 but not for G-3. The latter glucan emitted no fluorescence while G-2 showed high fluorescence intensity confirming its $(1\rightarrow3),(1\rightarrow6)\text{-}\beta$ configuration. G-1 emitted significantly lower fluorescence indicating traces of $(1\rightarrow3)\text{-}\beta\text{-D-glucans}$.

All the isolated glucans were able to inhibit HMGCR *in vitro* and therefore, they might impair the cholesterol biosynthetic pathway. They also showed immunomodulatory activities although they did not induce significant variations on TNF- α secretion by LPS-activated THP-1/M cells, they manage to lower IL-1 β and

IL-6 secretion. The glucans showed cytotoxic effects toward tumoral breast cells but they did not interfere with normal breast cells growth. The G-3 fraction showed the highest cytotoxic activity, reducing 73% viability after 48h of incubation, when analyzed by MTT. The cytotoxic effect of G-3 was also observed when the cells treated with the glucan were analyzed by Live/Dead® Viability kit. However, it was observed that G-3 was unable to scavenge DPPH radical when applied at similar concentrations than G-1 and G-2 fractions. The results observed on this study suggest promising biological activities for the purified fractions and moreover, they demonstrated that different chemical structures observed for each glucan, such as α/β -configuration and branching degree may highly influence their solubility, tridimensional conformation and also their interaction with cells and consequently provide different biological outcomes. The most significant differences on the biological effects exerted by the three glucans were observed on the scavenging activity and the inhibition of tumor cell growth. Further investigations should be carried out to confirm if this difference may be related to chemical structure or physical conformation.

Acknowledgements

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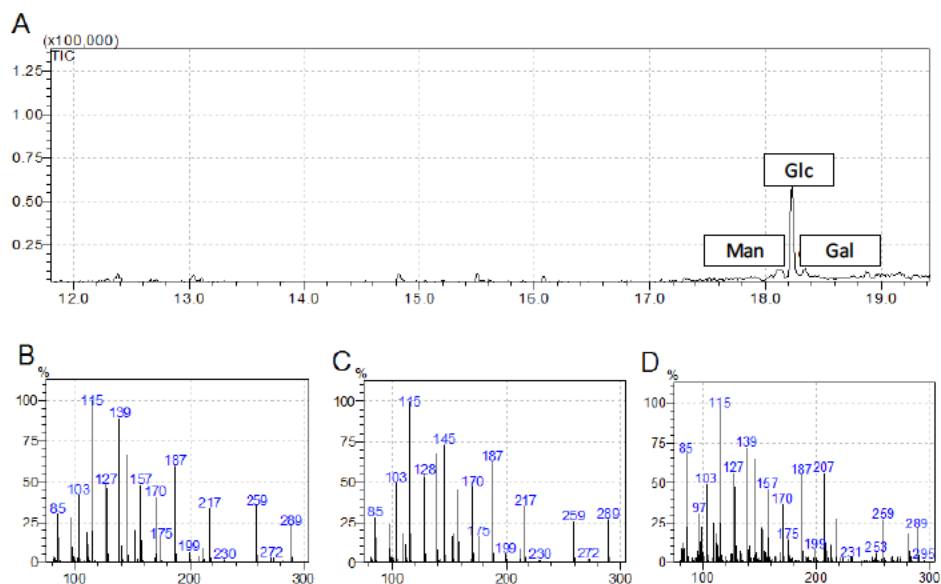
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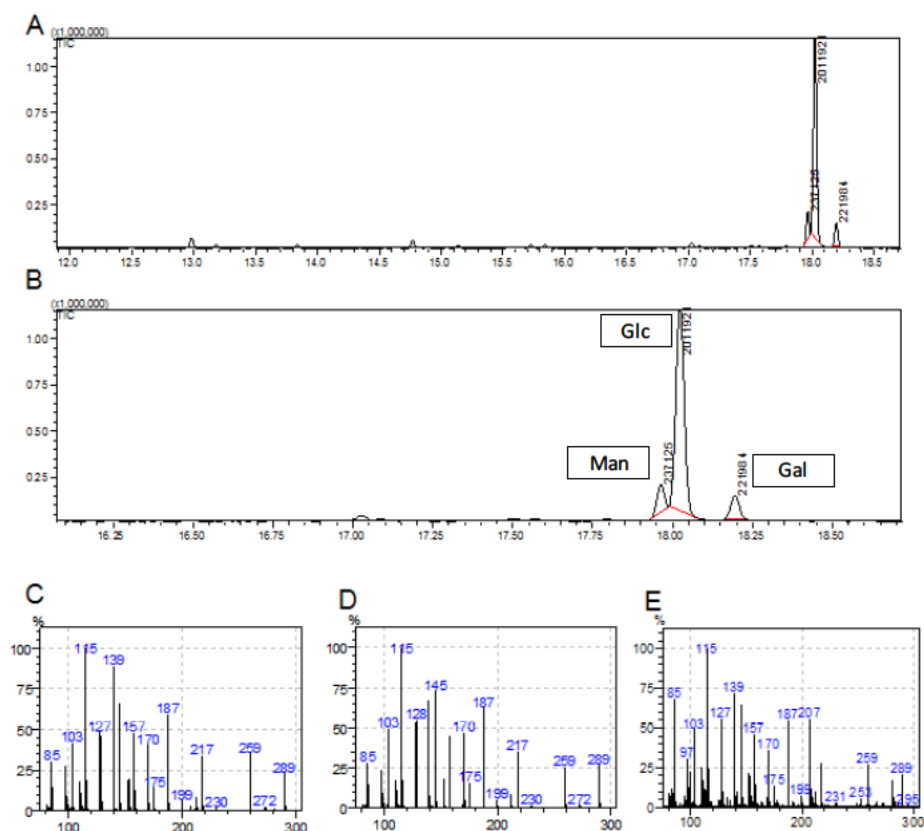
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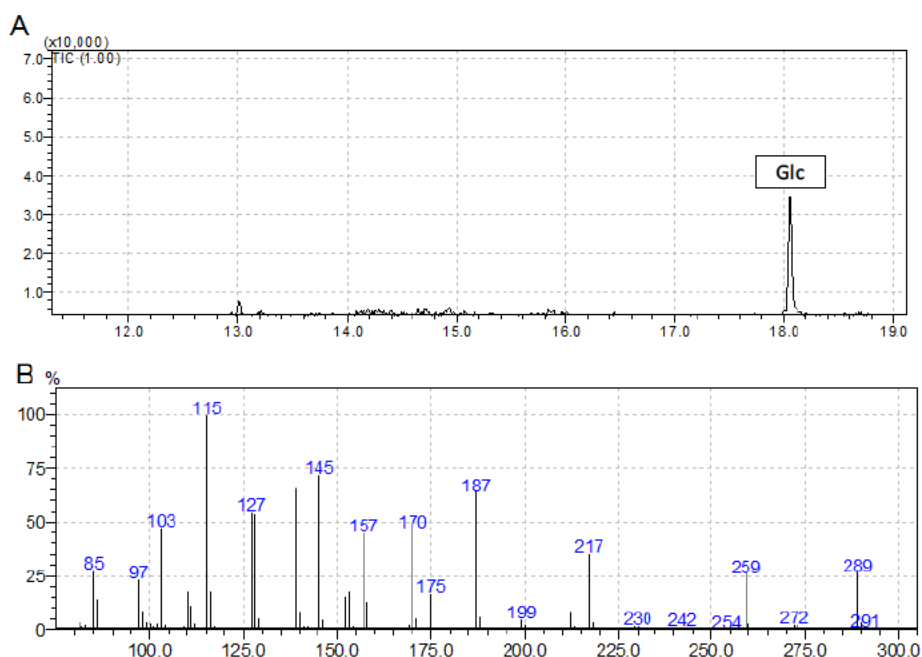
Supplementary material



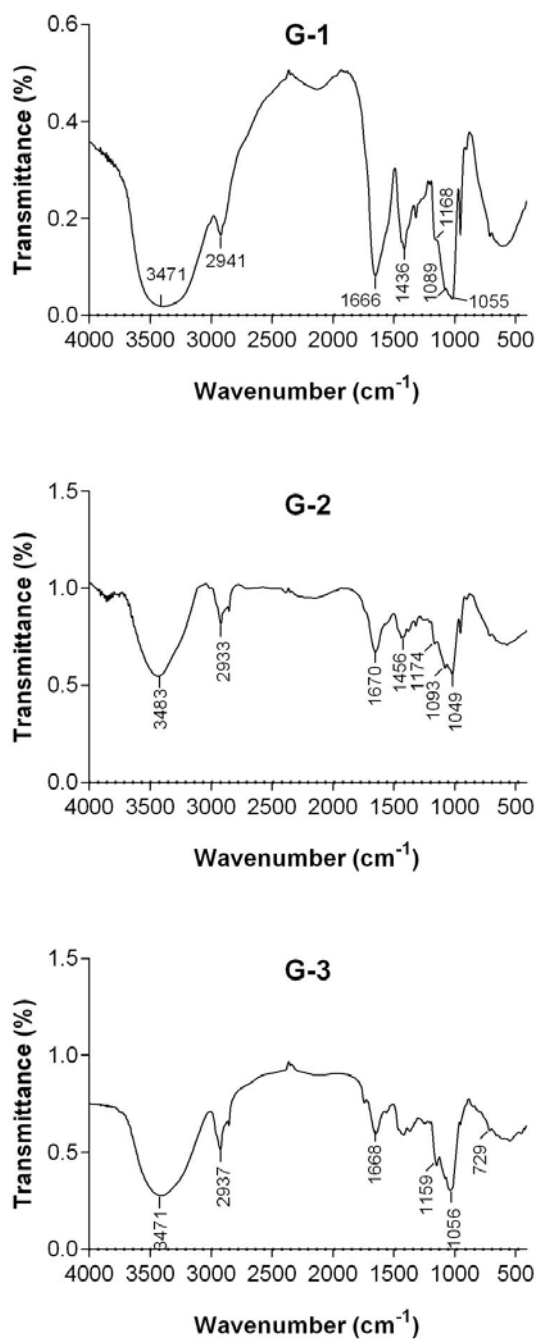
Supplementary Figure 1. (A) GC-MS chromatogram of alditol acetates obtained after acid hydrolysis, NaBH_4 reduction and acetylation of G-1. The retention time of Mannose 18.125 min (B), Glucose 18.230 min (C) and Galactose 18.345 min (D) and their respective electron impact profile (at m/z 75 to 300) can be observed. The analysis was performed with SH-RTX-5 ms at 280 °C. Electron ionization at 70 eV.



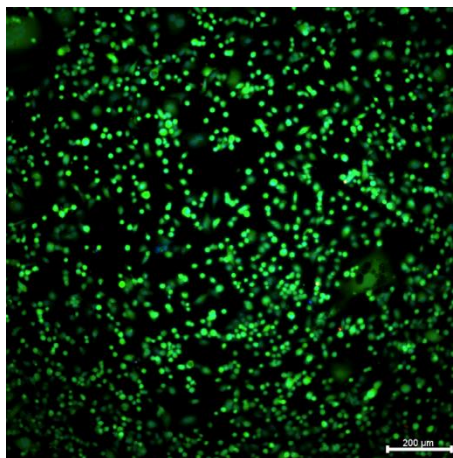
Supplementary Figure 2. (A) GC-MS chromatogram and zoom view (B) of alditol acetates obtained after acid hydrolysis, NaBH₄ reduction and acetylation of G-2. The retention time of Mannose 17.960 min (C), Glucose 18.030 min (D) and Galactose 18.190 min (E) and their respective electron impact profile (at m/z 75 to 300) can be observed. The analysis was performed with SH-RTX-5 ms at 280 °C. Electron ionization at 70 eV.



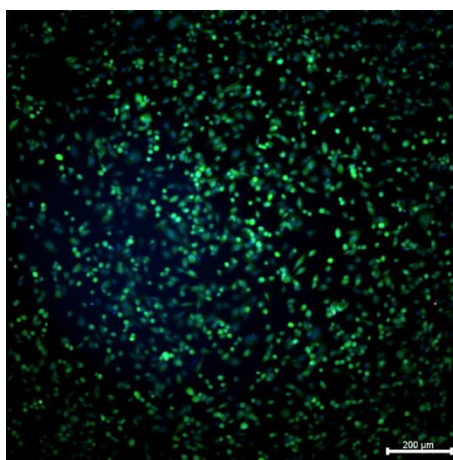
Supplementary Figure 3: (A) GC-MS chromatogram of alditol acetates obtained after acid hydrolysis, NaBH_4 reduction and acetylation of G-3. The retention time of Glucose 18.130 min (B) and its electron impact profile (at m/z 75 to 300) can be observed. The analysis was performed with SH-RTX-5 ms at 280 °C. Electron ionization at 70 eV.



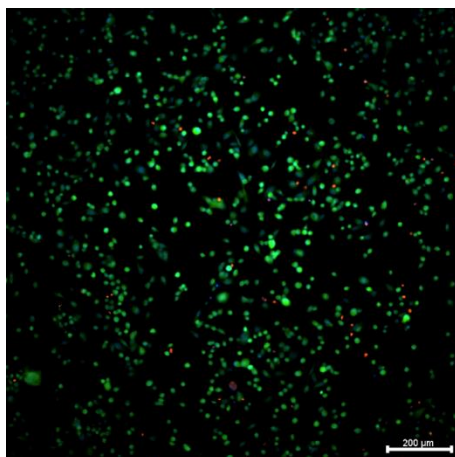
Supplementary Figure 4. FT-IR spectra of G-1, G-2 and G-3 fractions.



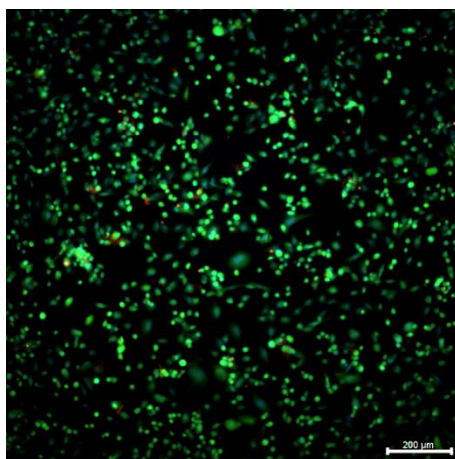
Supplementary Figure 5. MDA-MB-231 cells after 24 h of incubation with the vehicle control and addition of Live/Dead® Viability/Cytotoxicity kit. Blue fluorescence: cell nuclei; green fluorescence: live cells; and red fluorescence: dead cells. Pictures were taken by In Cell Analyzer.



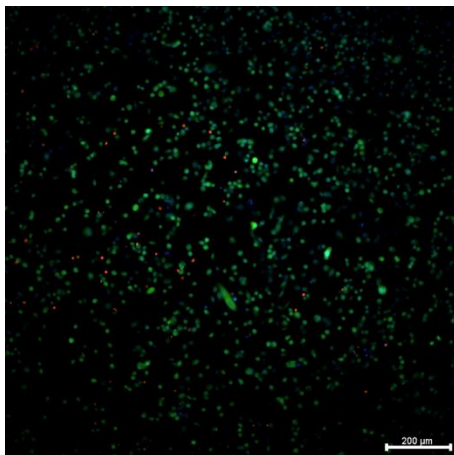
Supplementary Figure 6. MDA-MB-231 cells after 48 h of incubation with the vehicle control and addition of Live/Dead® Viability/Cytotoxicity kit. Blue fluorescence: cell nuclei; green fluorescence: live cells; and red fluorescence: dead cells. Pictures were taken by In Cell Analyzer.



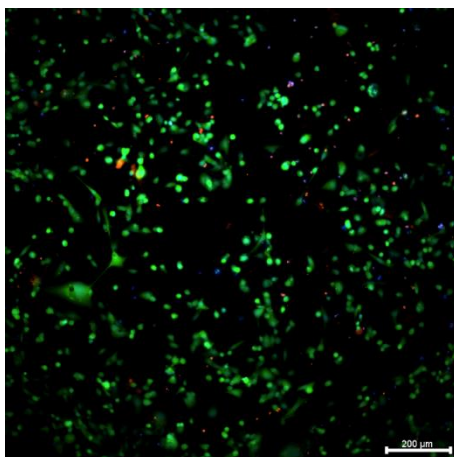
Supplementary Figure 7. MDA-MB-231 cells after 24 h of incubation with G-2 (50 µg/mL) and addition of Live/Dead® Viability/Cytotoxicity kit. Blue fluorescence: cell nuclei; green fluorescence: live cells; and red fluorescence: dead cells. Pictures were taken by In Cell Analyzer.



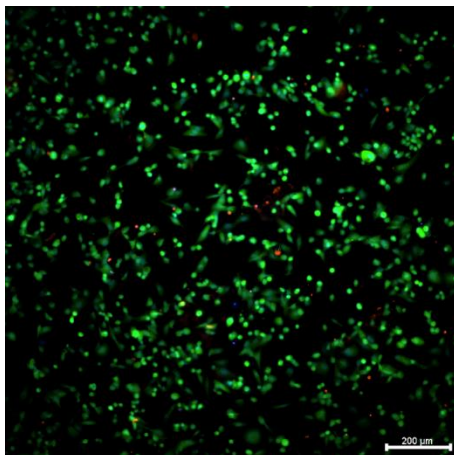
Supplementary Figure 8. MDA-MB-231 cells after 48 h of incubation with G-2 (50 µg/mL) and addition of Live/Dead® Viability/Cytotoxicity kit. Blue fluorescence: cell nuclei; green fluorescence: live cells; and red fluorescence: dead cells. Pictures were taken by In Cell Analyzer.



Supplementary Figure 9. MDA-MB-231 cells after 24 h of incubation with G-2 (250 µg/mL) and addition of Live/Dead® Viability/Cytotoxicity kit. Blue fluorescence: cell nuclei; green fluorescence: live cells; and red fluorescence: dead cells. Pictures were taken by In Cell Analyzer.



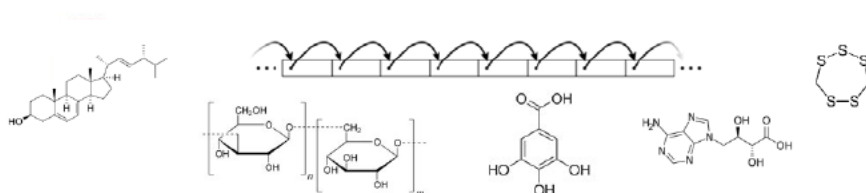
Supplementary Figure 10. MDA-MB-231 cells after 24 h of incubation with G-3 (250 µg/mL) and addition of Live/Dead® Viability/Cytotoxicity kit. Blue fluorescence: cell nuclei; green fluorescence: live cells; and red fluorescence: dead cells. Pictures were taken by In Cell Analyzer.



Supplementary Figure 11. MDA-MB-231 cells after 48 h of incubation with G-3 (250 $\mu\text{g/mL}$) and addition of Live/Dead® Viability/Cytotoxicity kit. Blue fluorescence: cell nuclei; green fluorescence: live cells; and red fluorescence: dead cells. Pictures were taken by In Cell Analyzer.

Chapter 4

Sequential procedure to scale up the production of functional ingredients



Preface

The previous works grouped in Chapters 1, 2 and 3 described the use of advanced methodologies to obtain bioactive ingredients from shiitake mushrooms but also more simple protocols that can be more easily up scaled. The latter might be more convenient for the industry since the necessary equipment is less expensive. However, large amounts of raw material are necessary to extract each type of compound making the production of several functional ingredients (with different chemical composition) less economically profitable. Moreover, the different production lines might leave large amounts of waste that would need further revalorization.

Therefore, a novel system was studied to extract fractions containing bioactive compounds of different chemical nature from the same batch of shiitake powder. The designed protocol was simple, scalable and reduced the amount of raw material utilized. The work entitled *Extraction of bioactive compounds against cardiovascular diseases from Lentinula edodes using a sequential extraction method* described a sequential procedure, combining three environmentally-friendly methods (using water, supercritical CO₂ and hot water extractions) to obtain different fractions containing specific compounds from shiitake mushrooms with interesting properties against cardiovascular diseases. Bioactive molecules such as β -D-glucans, chitins, hypotensive peptides, eritadenine, lenthionine, ergosterol, antioxidant phenols, etc. were determined in each extracted fraction and their biological activities were studied *in vitro* to confirm that the consecutive processing did not affect their beneficial properties.

The novelty of the proposed methodology was the use of the residue obtained after one extraction as starting material to extract other interesting compounds. Moreover, the proposed design showed another advantage: the by-products generated after harvesting of fruiting bodies (such as the lower part of the mushroom stipes that are usually cut and discarded) could also be used as raw material to obtain the same bioactive compounds. Therefore, the sequential procedure could be used to valorize those cultivation wastes.

Manuscript 1

Extraction of bioactive compounds against cardiovascular diseases from *Lentinula edodes* using a sequential extraction method

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Abstract

Three extraction methods were sequentially combined to obtain fractions from *Lentinula edodes* (shiitake mushrooms) containing bioactive compounds against cardiovascular diseases (CVDs). Fruiting bodies were first extracted with plain water, obtained residue was then submitted to supercritical fluid extraction (SFE) and remaining residue submitted to hot water extraction. Sequential design allowed reutilization of the non-extracted material as raw material for the successive extractions increasing extraction yields and separating interesting compounds. Obtained fractions contained different amounts of β -D-glucans, chitins, eritadenine, lenthionine, ergosterol, proteins/peptides and phenolic compounds conferring them different bioactivities. Water soluble fractions showed high antioxidant activities (ABTS⁺• and DPPH• scavenging capacity and reducing power), they were also able to inhibit one of the main enzymes involved in hypertension (angiotensin-I converting enzyme) and the key enzyme of cholesterol metabolism (3-hydroxy-3-methylglutaryl coenzyme A reductase). The latter inhibitory activity was also noticed in SFE extracts although ergosterol and other lipid-like molecules were isolated. Dietary fibers were separated in the third extraction. Therefore, with this sequential extraction procedure bioactive compounds against CVDs can be selectively separated from a single batch of shiitake powder.

Introduction

Cardiovascular diseases (CVDs) are still one of the leading causes of mortality in developed countries. CVDs are associated with multiple factors such as high triglycerides and low density lipoprotein cholesterol (LDLc) levels, LDL oxidation, increased platelet aggregation, hypertension and smoking [1, 2].

Nowadays, functional foods containing phytosterols or β -D-glucans are being commercialized to reduce low to moderate hypercholesterolemia because these compounds showed the ability of impairing exogenous cholesterol absorption. Hypotensive foods are also marketed containing specific peptides able to inhibit the angiotensin I converting enzyme (ACE) [3] and many juices and functional drinks indicate that they contain many compounds with high antioxidant properties. These compounds might inhibit LDL oxidation, prevent atheroma plaque formation, and so forth, contributing to reduce the risk of CVDs. However, a more effective novel food may be designed if all compounds are combined and, besides those inhibiting cholesterol absorption, other compounds are incorporated such as those reducing the risk of CVDs by different mechanisms of action [4].

Edible mushrooms are natural sources of hypocholesterolemic compounds, such as fungal sterols (ergosterol and derivatives) and polysaccharides (β -D-glucans, chitins, etc.) that according to *in vitro* experiments might inhibit cholesterol absorption and biosynthesis [4-7]. Eritadenine (2(R),3(R)-dihydroxy-4-(9-adenyl) butanoic acid) from *Lentinula edodes* (shiitake mushrooms) was also able to lower cholesterol levels by acting as an inhibitor of the S-adenosyl-L-homocysteine hydrolase involved in the hepatic phospholipid metabolism [8,9]. When lard was supplemented with extracts containing these compounds and fed to mice, they succeeded to avoid the increasing of cholesterol levels noticed in control mice fed only with lard [10].

Moreover, water extracts obtained from edible mushrooms such as *Hypsizygus marmoreus* and *Lactarius camphorates* were also able to inhibit ACE because of their hypotensive peptides [11,12] and lenthionine (1,2,3,5,6-pentahiepane), an organosulfur compound responsible for the characteristic flavor of shiitake mushrooms, inhibited platelet aggregation [13]. Water and hot water extracts

from other mushroom species also showed interesting antioxidant activities that correlated to their levels of ergothioneine and phenolic compounds [14-16].

Several methods to isolate or extract each type of those previously mentioned compounds have been already reported. Different β -D-glucans types are usually isolated by alkali/acid treatments, hot water (120 °C, 20 min), microwave, pressurized solvent or ultrasound assisted extractions [17-19]; chitins needed more drastic treatments [20]; and sterols were isolated by using mixtures of organic solvents or supercritical CO₂ extractions [21]. However, all these methods were optimized to use mushrooms as raw material and for each extraction, a new batch of powdered mushrooms should be used. In the present work, a sequential extraction method was designed to extract from the same batch of mushroom powder a few fractions enriched in different bioactive compounds against CVDs. The residue remaining after one extraction was used to extract other compounds at the following extraction step instead of using different batches of mushroom powder for each type of compound.

Materials and methods

Biological material

Dry *Lentinula edodes* S. (Berkeley) mushrooms were obtained in a local market (Madrid, Spain). Fruiting bodies were ground as a whole or divided into caps and stipes until a fine powder was obtained and stored at -20 °C as indicated by Ramirez-Anguiano et al. (2007) [16] (they were used to analyze separated tissues). Larger amounts of powdered shiitake mushrooms were also purchased from Glucanfeed S.L. (La Rioja, Spain). Obtained powder showed a particle size lower than 0.5 mm, and moisture content lower than 5% and it was stored in darkness at -20 °C until further use.

Reagents

Solvents as hexane (95%), chloroform (HPLC grade), methanol (HPLC grade), acetonitrile (HPLC grade) were obtained from LAB-SCAN (Gliwice, Poland) and absolute ethanol, sodium carbonate (Na₂CO₃) and sulfuric acid (H₂SO₄) from Panreac (Barcelona, Spain). Potassium hydroxide (KOH), ascorbic acid, 2,6-Di-tert-

butyl-*p*-cresol (BHT), bovine serum albumin (BSA), acetylacetone, *p*-dimethylaminebenzaldehyde, Trizma base, HCl (37%), trifluoroacetic acid (99%), phenol, Folin Ciocalteu's phenol reagent, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), potassium persulfate, Angiotensin-I Converting Enzyme (ACE) (5 UN/mL), zinc chloride (ZnCl₂) solution (0.1 M) as well as hexadecane, ergosterol (95%), D-glucose, D-glucosamine hydrochloride, gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium phosphate dibasis dihydrate (NaH₂PO₄ · 2H₂O), sodium phosphate monobasic sodium phosphate dibasic anhydride (Na₂HPO₄), potassium ferricyanide (K₃[Fe(CN)₆]), ferric chloride (FeCl₃) and Trolox were purchased from Sigma-Aldrich Quimica (Madrid, Spain). CO₂ was supplied by Air-Liquid, S.A. (Madrid, Spain). D-Eritadenine (90%) was acquired from Sy Synchem UG & Co. KG (Felsberg, Germany), lenthionine (80%) from Cymit (Barcelona, Spain) and N-(2-aminobenzoyl)glycyl-4-nitro-L-phenylalanine (Abz-Gly-Phe(NO₂)-Pro) from Bachem Feinchemikalien (Bubendorf, Switzerland). All other reagents and solvents were used of analytical grade.

Sequential extractions

A method to obtain different bioactive fractions from powdered shiitake mushrooms was optimized based on three successive extractions (Figure 1). Firstly (Step A), mushroom powder was mixed with water (50 g/L) at room temperature (RT) and vigorously stirred during 1 minute. Afterwards, the mixture was centrifuged (7 min, 7000 rpm, 10°C) in a Heraus Multifuge 3SR+ centrifuge (Thermo Fisher Scientific, Madrid, Spain). Obtained supernatant (considered extract ExA) was separated from the residue (ReA) and both fractions were freeze dried in a LyoBeta 15 lyophilizer (Telstar, Madrid, Spain).

Secondly (Step B), freeze-dried ReA (253 g) was ground, sieved until particles size < 0.5 mm and submitted to supercritical fluid extraction (SFE). ReA was mixed with 1.9 kg of 5 mm diameter stainless steel spheres (a ratio 1:1 (v/v) extract:spheres) in a 2L extraction cell connected to a SFE pilot-scale plant (model SF2000, TharTechnology, Pittsburgh, PA, USA). Pressurized CO₂ was forced to reach supercritical state and injected in the loaded extraction cell. Extracted material was collected in two different separators (S1 and S2) each of 0.5 L capacity with

independent control of temperature and pressure. The extraction vessel had a ratio of 5.5 height/diameter (a detailed explanation of the experimental device can be found at Garcia-Risco et al. (2011)[22]). Extraction was carried out at 35 MPa and 40 °C. Pressure of S1 and S2 was maintained at 10 and 6 MPa respectively and temperature in both of them was 40 °C. The CO₂ flow was set at 3.6 kg/h and during the total extraction time (3 h) and it was recirculated. Extracted compounds were precipitated in both separators and at the end of the extraction process, the fractions were dragged with ethanol and immediately submitted to concentration until dryness on a rotary vacuum evaporator. Dried extract (ExB) was stored at -20 °C until further analysis and non-extracted residual material (ReB) was separated from steel spheres by sieving.

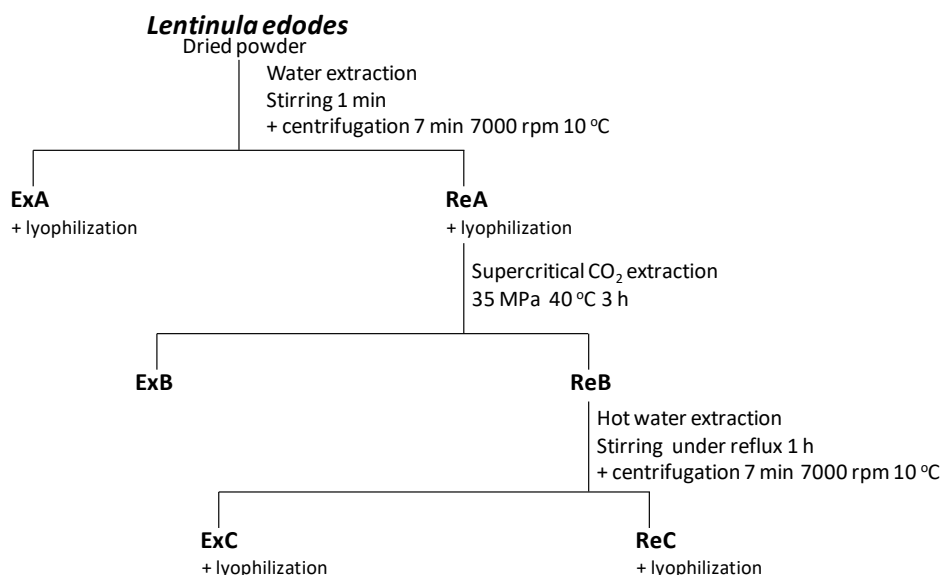


Figure 1. Outline of the sequential extraction method utilized to obtain different extracts from shiitake mushrooms.

Finally (Step C), ReB (100 g/L) was submitted to hot water extraction (98 °C) in a 0.5 L flask during 1 hour under vigorous stirring and reflux using a glass steam condenser. The soluble (ExC) and non-soluble (ReC) fractions were separated by centrifugation (7 min, 7000 rpm, 10 °C) and freeze dried.

Carbohydrates determinations

The total carbohydrate content of shiitake mushroom samples or obtained extracts was determined by the phenol-sulphuric acid method adapted from Dubois et al. (1956) [23]. Briefly, samples (1 mg) were mixed with 1 mL of MilliQ water and stirred during 2 min. The mixtures (25 μ L) were added to a 96-well plate, plus 25 μ L of 5% phenol solution (w/v) and 125 μ L of concentrated H₂SO₄. Afterwards, the plate was sealed and incubated in a water bath at 80 °C for 30 min. Samples absorbance was read using a M200 Plate Reader (Tecan, Mannedorf, Switzerland) at 595 nm. A standard curve of D-glucose (0.032 to 0.8 mg/mL) was used for quantification.

Chitin content was determined according to Smiderle et al. (2017) [18]. Firstly, samples were hydrolyzed with 6 M HCl at 100 °C for 2 h and adjusted to pH 10.0 after cooling down. Then, hydrolyzed samples (250 μ L) were treated as described by Rementeria et al. (1991) [24]. Samples absorbance was measured at 530 nm using an Evolution 600 UV-vis (Thermo Fisher Scientific, Spain) spectrophotometer. A glucosamine hydrochloride standard curve was used for quantification.

The β -D-glucan content of the obtained mushroom samples and extracts (50 mg) was evaluated by a β -D-glucan determination kit specific for mushrooms and yeasts (Megazyme®, Biocom, Barcelona, Spain) following the instructions of the user's manual.

Total phenol content and soluble protein or peptides determination

The total phenol concentration of samples (10 mg) was determined by the Folin-Ciocalteu method according to the procedure of Ramirez-Anguiano *et al.* (2007) [16]. Gallic acid was used as standard for quantification.

The total soluble protein concentration of the samples (10 mg/mL) was determined using the Bradford method reagents (Sigma-Aldrich, Madrid, Spain) according to the Instruction Manual. To determine the amount of peptides, extracts were solubilized in water (100 mg/mL) and submitted to centrifugation (14000 rpm, 30 min) using Amicon Ultra filter devices with Ultracel 3K membrane (Millipore,

Billerica, USA) obtaining a filtrate (< 3 kDa) and a concentrate (> 3 kDa). The latter fraction was submitted to a second centrifugation (14000 rpm, 20 min) using Nanosep centrifugal devices with Omega 10K membrane (Pall Life Sciences, New York, USA), obtaining a filtrate (fraction with MW between 3 and 10 kDa) and a concentrate (> 10 kDa). The obtained fractions were freeze dried and mixed with the Bradford reagent as described above. BSA was used as standard (0.0125 to 0.5 mg/mL) for protein quantification.

Eritadenine and lenthionine determination by HPLC-DAD

Eritadenine was extracted from the samples and analyzed following the procedure of Afrin et al. (2016) [25] with modifications. Briefly, samples (1 g) were mixed with 10 mL of 60% ethanol (v/v) and stirred for 2 min. The mixture was centrifugated (15 min, 7000 rpm, 10 °C) and the supernatant was collected. Afterwards, 10 mL of 60% ethanol (v/v) was added for a second extraction and both supernatants were pooled together and submitted to vacuum filtration. The filtrate was concentrated on a rotary vacuum extractor at 60 °C until dryness. Identification and quantification of eritadenine were carried out using a C18 Spherisorb ODS2 4 x 250 mm analytical column with a 5 µm particle size (Waters, Missisagua, Ontario, Canada) coupled to an HPLC system (Pro-Star 330, Varian, Madrid, Spain) with PDA detector (Pro-Star 363 module, Varian, Madrid, Spain). Samples were dissolved in mobile phase (5 mg/mL) and they were injected (10 µL) and developed under a constant flow (0.5 mL/min) and an isocratic mobile phase of water:acetonitrile (98:2, v/v 1% TFA). Eritadenine was quantified at 260 nm using a commercial standard. The compound eluted at 11.6 min and showed the characteristic eritadenine UV-spectrum.

Lenthionine determination was carried out according to the procedure of Hiraide et al. (2010) [26] with slight modifications. Basically, samples (50 mg) were mixed with 1 mL of 0.2 M Tris-HCl buffer (pH 8.0) and stirred for 1 h. Afterwards, methanol (0.5 mL) was added, stirred for 2 min and centrifuged (14000 rpm 5 min). The obtained supernatant was collected and the residue submitted to extraction twice. Supernatants were pooled together, diluted with 2.5 mL MilliQ water and filtered

using a syringe through a 0.45 μm pore size filter. The filtrate was applied to an ODS cartridge (Waters, Mississauga, Ontario, Canada) preactivated with methanol following manufacturer's instructions. The cartridge was washed with 1 mL 30% methanol (v/v) and lenthionine was eluted with 1 mL 65% methanol (v/v). Obtained eluate (50 μL) was injected into an HPLC-DAD system (the same column and equipment than above described) and developed using an isocratic mobile phase (65% methanol v/v), a constant flow (0.7 mL/min) and temperature (45 $^{\circ}\text{C}$). Lenthionine (retention time 10.4 min) was quantified at 230 nm and identified using a lenthionine commercial standard.

Ergosterol determination by GC-MS-FID

Fungal sterols from samples were evaluated following the procedure described by Gil-Ramirez et al. (2013) [21]. The unsaponified fractions obtained (6 mg/mL) were injected into an Agilent HP-5ms capillary column (30 m x 0.25 mm i.d. and 0.25 μm phase thickness). The column was connected to a 7890A System gas chromatograph (Agilent Technologies, Santa Clara, CA, USA), comprising a split/splitless injector, an electronic pressure control, a G4513A autoinjector, a 5975C triple-axis mass spectrometer detector and a GC-MS Solution software. The injector and detector conditions as well as the column temperature program were those described by Gil-Ramirez et al. (2013) [21]. Ergosterol was used as standard and hexadecane (10% v/v) as internal standard for quantification.

Antioxidant activities

Mushroom powder and obtained extracts were dissolved in water (0.02 to 0.5 mg/mL) and assayed for their ABTS⁺ scavenging activity assay. ABTS radical was chemically generated using potassium persulfate and ABTS⁺ scavenging activity was analyzed spectrophotometrically according to Re et al. (1999) [27], measuring changes in absorbance (734 nm) at several concentrations after 15 minutes of incubation at room temperature in darkness. Similarly, samples were also dissolved in methanol (0.02 to 0.5 mg/mL) and mixed with DPPH[•] (76 μM) to determine their scavenging capacity according to Mau et al. (2001) [28]. Absorbance at 517 nm was recorded at several concentrations after 15 min incubation at room temperature in

darkness. For both radicals the IC_{50} was established using the linear correlation obtained with increasing sample concentrations and compared with Trolox to express the results as their TEAC values (trolox equivalent antioxidant capacity).

The ferric ion reducing power of the extracts was evaluated according to the method of Oyaizu (1986) [29]. Samples were dissolved in 200 mM sodium phosphate buffer (0.1 to 10 mg/mL) and treated as described by Mau et al. (2005) [30]. Absorbance increase was recorded at 700 nm using several concentrations to estimate their EC_{50} . Afterwards, it was expressed as TEAC values to be able to compare with the other antioxidant activities.

HMGCR inhibitory activity

The obtained extracts were solubilized in water, ethanol:water (1:4) or assay buffer (50 mg/mL) and applied (20 μ L) into a 96-wells plate. Their inhibitory activity was measured using the commercial HMGCR (3-hydroxy-3-methylglutaryl coenzyme A reductase) activity assay (Sigma-Aldrich, Madrid, Spain) according to the manufacturer's instructions by monitoring their absorbance change (340 nm) at 37 °C using a 96-wells microplate reader BioTek Sinergy HT (BioTek, Winooski, USA). Pravastatin was utilized as a control for positive inhibition.

ACE inhibitory activity

The Angiotensin-I Converting Enzyme (ACE) inhibitory activity of the obtained extracts was evaluated using the fluorimetric method described by Sentandreu & Toldra (2006) [31] with slight modifications. Basically, 40 μ L of different samples dilutions were added to a 96-wells plate, followed by addition of 160 μ L of Abz-Gly-Phe(NO₂)-Pro (0.45 mM) and 40 μ L of ACE working solution (0.04 U/mL). The plate was incubated during 1 h at 37 °C measuring the generated fluorescence with excitation and emission wavelengths respectively 355 and 405 nm. Milli Q water was utilized as a control for negative inhibition.

Statistical analysis

Differences were evaluated at a 95 % confidence level ($p \leq 0.05$) using a one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison test. Statistical analysis was performed using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA, USA). Correlation between different variables was evaluated by computing Pearson correlation coefficient and determination coefficient ($P \leq 0.05$).

Results and discussion

Selection of the starting material

The distribution of compounds with beneficial activities against CVDs within the *L edodes* fruiting body was investigated as a preliminary study to elucidate whether it was more convenient to use the complete mushroom or perhaps only caps or stipes as raw material for the extractions. However, when the amount of the main bioactive compounds was determined (Table 1), no significant differences were noticed between tissues. They contained approx. 40% (w/w) carbohydrates and most of them were β -D-glucans since chitins were found in lower concentrations. Ergosterol and the other bioactive compounds showed levels similar to previous studies [16, 25, 32] being lenthionine the compound found in the lowest concentration (0.15 mg/g) but also similar to other publications [13, 26].

Table 1 (Part A). Total carbohydrates (TC), β -D-glucans (β G), chitins (CH) and proteins (PR) levels of shiitake fruiting bodies and two separated tissues (indicated values are w/w). No significant differences ($P \leq 0.05$) were found between different samples for the same component.

	TC (g/100g)	β G (g/100g)	CH (g/100g)	PR (g/100g)
Whole	40.67 \pm 0.65	29.32 \pm 1.04	6.03 \pm 0.29	13.42 \pm 0.22
Cap	39.72 \pm 2.42	29.89 \pm 1.55	5.96 \pm 0.87	13.81 \pm 0.12
Stipe	42.37 \pm 1.42	32.01 \pm 1.72	6.28 \pm 1.09	12.90 \pm 0.08

Table 1 (Part B). Eritadenine (EA), lenthionine (LT), ergosterol (ER) and total phenolic compounds (PH) levels of shiitake fruiting bodies and two separated tissues (indicated values are w/w). No significant differences ($P \leq 0.05$) were found between different samples for the same component.

	EA (mg/g)	LT (mg/g)	ER (mg/g)	PH (mg/g)
Whole	1.43±0.13	0.15±0.01	2.40±0.02	9.27±0.05
Cap	1.13±0.01	0.13±0.01	2.24±0.04	9.68±0.50
Stipe	1.12±0.11	0.11±0.01	2.32±0.05	8.95±0.43

The lower part of *L. edodes* stipes (the one is in contact with the cultivation substrate) is considered as a by-product and it is usually discarded during harvesting and not commercialized. However, the stipes showed similar concentrations than the complete fruiting body not only of the bioactive molecules but also of other nutrients such as proteins or carbohydrates. This observation indicated that the wasted stipes could be used as source of bioactive compounds as well as the complete fruiting bodies being an alternative for valorization of these wastes. Nevertheless, since the fruiting bodies were more easily available in larger concentrations the following experiments were carried out using the complete mushrooms.

Sequential extraction yields

The sequential extraction method was designed to optimize the use of shiitake mushrooms or their by-products (as they showed similar composition) as material to extract bioactive compounds. The idea was to consecutively use the residue of one extraction to carry out the next one in order to maximize the number of compounds that could be selectively isolated in each step avoiding the use of larger amounts of mushroom powder for independent extractions.

Water extraction (at room temperature) was selected as first extraction step because some of the bioactive compounds that could be solubilized in this medium were not thermostable (see later for details). Then, the lipid fraction could be extracted with SFE using moderate temperatures (as second step) and in the third step

hot water could be used to extract heat resistant molecules such as dietary fibers improving the intestinal bioavailability (for specific β -D-glucans) and making them more accessible for colonic microbiota [33,34].

With the first extraction, water extracted 37 g from 100 g (w/w) of the mushroom powder (Figure 2). This method was previously used to obtain water-soluble polysaccharides [35] and other compounds of lower molecular weight [36]. The lowest yield (1.1 %) was obtained with supercritical CO₂ extraction of the generated residue (1.7% of ReA) since it mostly extracted lipophilic compounds (ExB) and mushrooms have a low lipid content. Moreover, supercritical CO₂ exhibit a high selectivity enabling high recoveries of specific fatty acids, sterols and derivatives from fungal or other matrices [37]. Nevertheless, the obtained yield was similar to those described in previous publications where SFE extractions were carried out directly from fruiting bodies (Chapter 1, Manuscript 1) [38] indicating that SFE extractions could be also carried out using the residues obtained after the first extraction step.

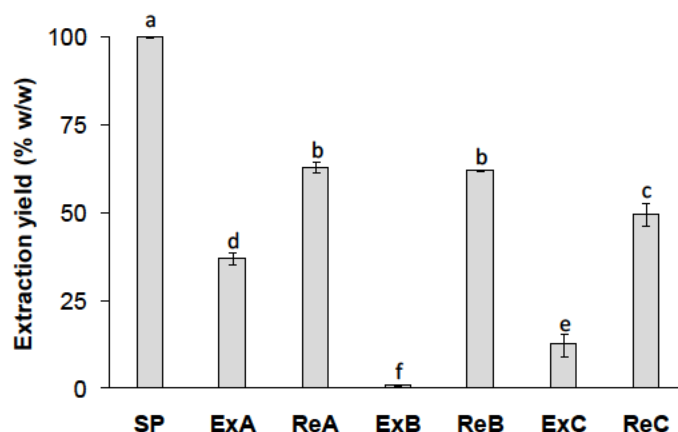


Figure 2. Extraction yields obtained after each step of the sequential extraction method expressed as percentage (w/w) referred to the initial amount of shiitake powder (SP). Different letters (a-f) showed statistical significance ($P \leq 0.05$) between different fractions.

The last step, a hot water extraction (98 °C), generated a fraction (ExC) containing 20 % (w/w) of ReB (12 % of the initial mushroom powder) leaving still a

large insoluble fraction (ReC) encompassing 49.5% of the starting material. This procedure is usually utilized to extract certain polysaccharides from the rest of non-soluble dietary fibres that remain in the residual fraction [39, 40].

Distribution of bioactive compounds within the obtained fractions

The levels of the different fungal polysaccharides were evaluated in all the obtained fractions. Results indicated that the water insoluble fractions contained higher levels of total carbohydrates, β -D-glucans and chitins than water-soluble or SFE fractions (Figure 3a). The mushroom powder contained 40.7% (w/w) total carbohydrates where 29.3% of them were β -D-glucans and 6.0% chitins suggesting that the remaining low percentage (approx. 5 %) should include oligosaccharides, sugars and perhaps small amounts of α -D-glucans or other heterosaccharides. Total carbohydrates values were in concordance with the literature [41]. β -D-glucans and chitins were slightly higher than some reports [42,43] but in the same range than others [44-46]. Differences might be due to the different methodologies utilized for their determination.

The water extract (ExA) contained a lower content of total carbohydrates and more than half of them were probably sugars and oligomers since they are easily solubilized in water and their β -D-glucan content was also lower than in the mushroom powder (10.8%). Although in low amounts, chitins were also detected but they might be degradation products or low molecular weight derivatives from chitins since the latter compounds are completely insoluble in water. These derivatives were also noticed in other reports [17,20] and they might be involved, together with the water soluble β -D-glucans, in the interesting biological activities noticed in mushroom water extracts. Water soluble β -D-glucans, α -D-glucans and fucomannogalactans were pointed as compounds potentially involved in the HMG-CoA reductase inhibitory activity noticed *in vitro* for water soluble extracts [36]. Moreover, chitin oligomers (water-soluble low molecular weight chitin (LMWC) and chitoooligosaccharides (COs) derivatives) could be involved in the hypocholesterolemic properties noticed for these extracts [47].

ReA and ReB showed a very similar carbohydrates profile including approx. 39-40% β -D-glucans and 7-8% chitins indicating that the supercritical CO₂ extraction did not significantly influenced the polysaccharide composition of the residue obtained after water extraction and none of them was extracted.

Alkaline/acid or hot water extractions are usually required to achieve fractions with high polysaccharides yields [35]. The hot water extraction did not succeed to separate β -D-glucans from chitins. The β -D-glucans extracted with hot water accounted for approx. less than half of the total carbohydrates noticed in ExC. As probably most of the oligosaccharides and monosaccharides were previously extracted in the ExA, the rest of carbohydrate content could be due to α -D-glucans or other heteropolysaccharides characteristic from *L. edodes* that were also extracted. Shiitake β -D-glucans such as lentinan are usually isolated using hot water extractions although their yields could be enhanced if alkaline media are used. However, alkalis were not recommended since they interfere with the lentinan 3D structure, essential for its biological activities [48]. Hot water soluble polysaccharides were suggested to decrease the oxidative damage related to hypercholesterolemia and modulating immune system [49-51].

Furthermore, approx. 2/3 of the carbohydrates present in the residue ReC were β -D-glucans, and chitins (10.2% w/w) that remained being part of the insoluble fractions. They were not extracted neither with cold/hot water or supercritical CO₂ since their concentrations in all the analyzed residues were almost the same. Their complex-forming capacity and polymeric structure are responsible for their extremely low solubility in many simple solvents [52]. However, this characteristic confers them the ability to act as dietary fibres remaining undigested in the intestine and acting on the colonic flora as prebiotic or as hypocholesterolemic fibers [47, 53].

The protein content measured in shiitake mushroom was in the range of previously reported studies since they might change from approx. 13 to 23 % depending on the cultivation conditions, developmental stage or commercial strain [54-56]. The highest protein content (Figure 3a) was obtained in ExA, indicating that a considerable amount of shiitake proteins might be easily extracted with cold water, probably because free proteins, peptides and amino acid derivatives are included in

this fraction. Indeed, in this fraction higher amounts of small proteins and peptides (8.57 mg/g) (with molecular weight (MW) between 10 and 3 kDa) than larger proteins (5.88 mg/g) (MW > 10 kDa) were noticed. It also contained a low amount of peptides and N-containing compounds with MW < 3kDa (0.83 mg/g). But, proteins might be also bound to polysaccharides as glycoproteins or proteoglycans impairing their extraction therefore, other proteins were found in the residues. The high pressure (and/or mild temperature) utilized during SFE extraction seemed to partially denature proteins since ReB protein levels were not the sum of those noticed in the fractions from the following extraction step (Table 2).

Table 2 (Part A). Distribution of bioactive compounds within the obtained fractions expressed as percentage of initial dry mushroom powder (taking into consideration the obtained yields). n.d. = not detected. ^{a-f}Different letters denote significant differences ($P \leq 0.05$) between different samples for the same component.

	TC	β G	CH	PR
	(g/100 g)	(g/100 g)	(g/100 g)	(g/100g SP)
SP	40.67±0.34 ^a	29.32±1.04 ^a	6.03±0.29 ^a	13.42±0.22 ^a
ExA	11.46±0.37 ^d	5.10±0.09 ^d	1.58±0.08 ^d	5.86±0.49 ^b
ReA	33.84±1.80 ^b	20.32±0.73 ^b	4.11±0.26 ^b	4.53±0.01 ^c
ExB	n.d. ^e	n.d. ^f	n.d. ^f	n.d. ^e
ReB	34.07±0.94 ^b	21.17±0.24 ^b	3.66±0.10 ^b	4.36±0.04 ^c
ExC	9.06±0.31 ^d	3.53±0.65 ^e	0.86±0.03 ^e	1.63±0.01 ^d
ReC	22.14±1.05 ^c	14.38±0.15 ^c	2.29±0.06 ^c	2.00±0.04 ^d

Table 2 (Part B). Distribution of bioactive compounds within the obtained fractions expressed as percentage of initial dry mushroom powder (taking into consideration the obtained yields). n.d. = not detected. ^{a-f}Different letters denote significant differences ($P \leq 0.05$) between different samples for the same component.

	EA	LT	ER	PH
	(mg/g)	(mg/g)	(mg/g)	(mg/g)
SP	1.43±0.13 ^a	0.15±0.01 ^a	2.40±0.02 ^a	9.27±0.05 ^b
ExA	1.46±0.14 ^a	0.09±0.01 ^b	0.03±0.01 ^e	10.35±0.01 ^a
ReA	0.32±0.01 ^{bc}	0.01±0.01 ^c	2.12±0.11 ^b	4.84±0.20 ^c
ExB	n.d. ^c	n.d. ^c	0.79±0.01 ^d	0.04±0.00 ^e
ReB	0.41±0.03 ^{bc}	n.d. ^c	1.08±0.01 ^c	4.33±0.10 ^c
ExC	0.57±0.03 ^b	n.d. ^c	0.06±0.01 ^e	2.91±0.01 ^d
ReC	0.17±0.00 ^c	n.d. ^c	1.01±0.11 ^{cd}	2.85±0.33 ^d

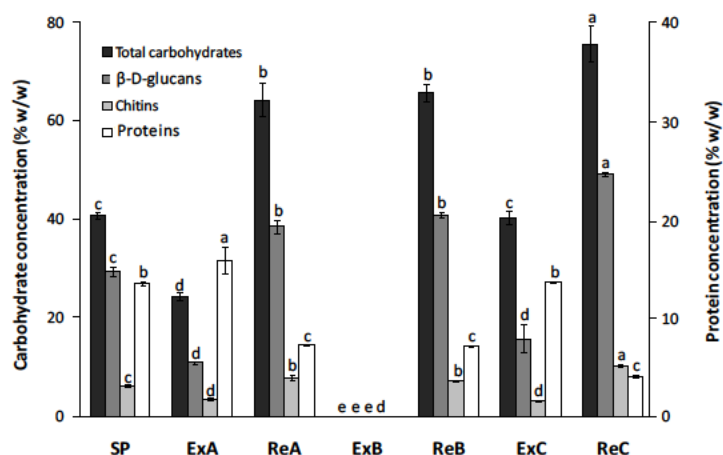


Figure 3. Distribution of total carbohydrates, β-D-glucans, chitins and proteins within the extracts obtained from shiitake powder expressed as concentrations within each fraction. Different letters (a-d) showed statistical significance ($P \leq 0.05$) between different samples for the same compound.

Nevertheless, the ExC obtained still contained high protein levels and only low amounts remained in ReC (Figure 3) suggesting that hot water enhanced extraction of many more proteins or different ones from those found in ExA. Nevertheless, the temperature selected might have also influenced proteins structure generating breaking down products from large (5.4 mg/g MW>10 kDa) and smaller proteins particularly those with MW between 10 to 3 kDa since the ExC contained lower content (1.84 mg/g) than ExA and the fraction with lower MW contained more peptides (1.74 mg/g MW <3 kDa).

Eritadenine is a water-soluble alkaloid therefore it was mainly extracted with cold water when compared with the amount noticed in the mushroom powder (Table 2). The minimal amount that remained in the residues was then almost completely extracted with hot water. These results also suggested that eritadenine was resistant to high temperatures and its extraction yield could be enhanced by increasing the extraction temperature. Eritadenine was 2.2 folds more concentrated in the ExA than in the shiitake powder (Figure 4a), amounts that were only slightly lower than those described in other studies obtained with more complex extraction procedures [25, 57].

Lenthionine was detected in shiitake powder at similar levels than previously reported [13,26]. It was also noticed in higher concentrations in the ExA extracts (Figure 4a). Afterwards, only traces remained in ReA but probably the slight increase of temperature together with the high pressure utilized for SFE extraction was sufficient to degrade it being undetected in the following extracted fractions. Lenthionine levels were drastically reduced when extraction temperature was higher than 80 °C [58,59]. Thus, apparently pressurized extractions made the compound more susceptible to degradation.

Ergosterol was mainly extracted with SFE (Table 2) obtaining a highly concentrated ExB extract (88.7 mg/g) (Figure 4b). This result was not surprising since previous works indicated that this lipophilic constituent that can be easily extracted with supercritical CO₂ [21, 60, 61]. Ergosterol in ExB represented 72% of total sterols since the extract also included other derivatives such as ergosta-7,22-dienol (18.5 mg/g), fungisterol (15.3 mg/g) and ergosta-5,7-dienol (1.5 mg/g). The supercritical extraction was carried out using steel spheres instead of sand as carrier material to

facilitate the subsequent ReB separation for further processing however, ergosterol yields were only slightly lower than usually obtained in similar extractions using sea sand (8.9 and 11.8 %, respectively).

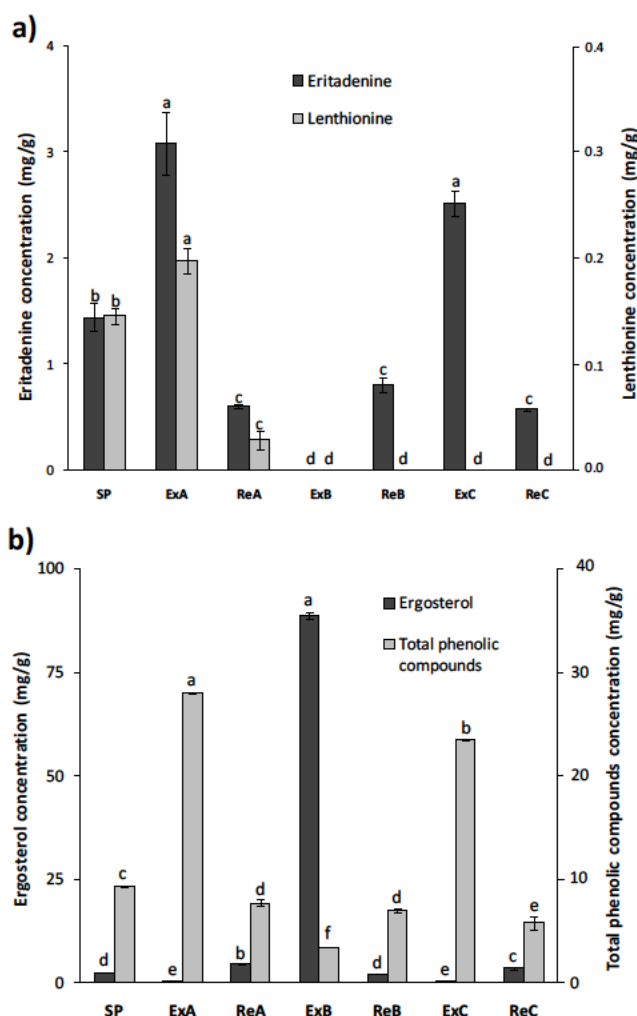


Figure 4. Distribution of a) eritadenine and lenthionine and b) ergosterol and phenolic compounds within the extracts obtained from shiitake powder expressed as concentrations within each fraction. Different letters (a-f) showed statistical significance ($P \leq 0.05$) between different samples for the same compound.

Total phenolic compounds were mostly found in the water extracts. Obtained fractions (ExA and ExC) contained respect. 3.0 and 2.5 fold more phenols than

initially detected in the mushroom powder. The second water extraction complemented the first one leaving in the last residue a very low amount of these compounds (Figure 4b). These results were in concordance with previous studies reporting that water achieved higher recoveries of total phenolic compounds in comparison with organic solvents such as methanol [16].

Biological activities of the obtained fractions

The antioxidant activity of the obtained fractions was evaluated as their ABTS^{•+} and DPPH[•] scavenging capacities and as their ferric ion reducing power. Results indicated that those fractions containing high concentrations of proteins, eritadenine and phenolic compounds showed high antioxidant activities (Figure 5). Their levels were more than double of the initial mushroom powder. ExA and ExC were the fractions with the lowest IC₅₀ values (respectively 0.05 and 0.06 mg/mL when using ABTS^{•+} and 0.15 and 0.17 mg/mL when using DPPH[•]) and the lowest EC₅₀ for their reducing powder (0.26 and 0.28 mg/mL). According to their TEAC values, the obtained extracts and residues showed higher affinity for the ABTS^{•+} radical than for DPPH[•].

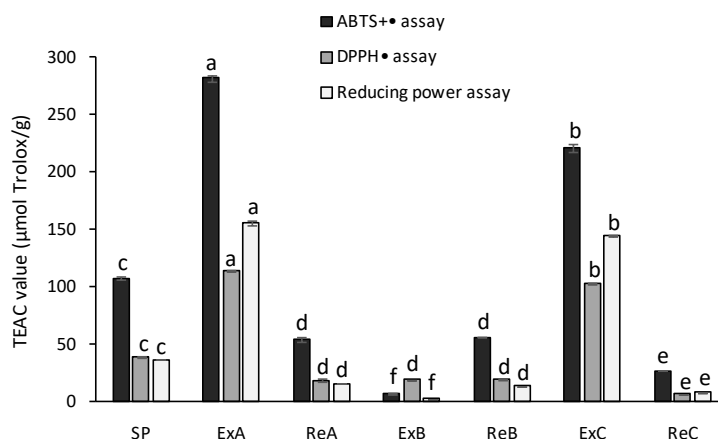


Figure 5. ABTS^{•+} and DPPH[•] scavenging capacity and ferric ion reducing power of shiitake powder and the fractions obtained after the sequential extraction method expressed as their TEAC value. Different letters (a-f) showed statistical significance ($P \leq 0.05$) between different samples.

The use of different solvents might also influence the obtained results since less polar compounds were extracted when methanol was used to carry out the DPPH[•] assay. The shiitake and ExA antioxidant activities were in concordance with previous studies [16, 62] where it was indicated that their antioxidant activities as radical scavengers were mainly due to the phenolic content and ergothioneine [14]. Similarly, the antioxidant activities noticed in the extracts also showed high correlations with their phenolic concentration (with $R^2 = 0.99$ for ABTS^{•+} and reducing power assays and 0.97 for DPPH[•] assays).

Cholesterol lowering in serum can be achieved via several mechanisms but mainly by impairing of exogenous cholesterol absorption or inhibiting endogenous cholesterol biosynthesis. The hypocholesterolemic activity of the obtained fractions was evaluated as their ability to inhibit the key enzyme of the cholesterol biosynthetic pathway (HMGCR) since the capacity to impair cholesterol absorption was already evaluated elsewhere for similar extracts containing high ergosterol levels (as ExB) and high β -D-glucans and dietary fibers contents (as ExC and ReC, respectively) [7, 17]. The initial shiitake powder showed a remarkable HMGCR inhibitory activity acting as pravastatin used as control (Figure 6).

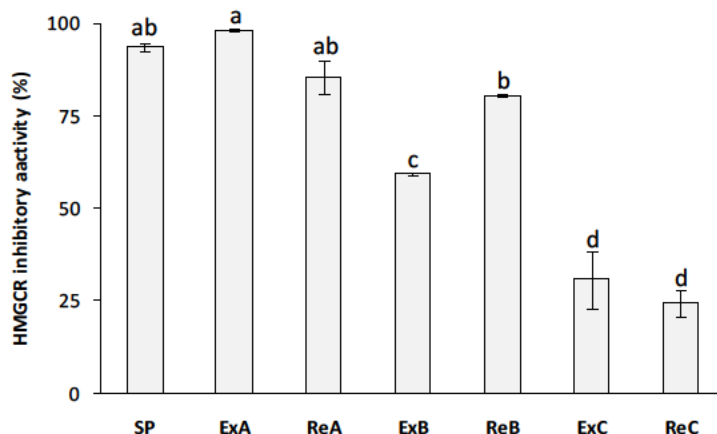


Figure 6. HMGCR inhibitory activity of shiitake powder and the fractions obtained after the sequential extraction method. Different letters (a-d) showed statistical significance ($P \leq 0.05$) between different samples.

ExA showed similar inhibitory activity (98 %) than the mushroom indicating that the responsible compounds might be water soluble. However, the inhibitors were not completely separated with a single cold water extraction since ReA and ReB were still able to lower HMGCR activity (respectively 86 and 80 %). Perhaps, to further improve the extraction yield of HMGCR inhibitors serial extractions with cold water could be carried out although this might involve dilution of the other bioactive compounds. Increase of the water temperature for extraction is not encouraged because after heat application, the inhibitory activity of the obtained fractions was drastically reduced to 31 and 24 % for ExC and ReC (respectively) suggesting that the compounds responsible for the inhibition were thermolabile. This indication was also noticed in previous studies [33]. The ExB extract was also able to inhibit 59% of the enzyme activity indicating that perhaps other compounds soluble in supercritical CO₂ (more lipophilic molecules) were also interfering with the HMGCR activity as was also previously noticed by Gil-Ramirez *et al.* (2013) [63] for SFE extracts obtained from *Agaricus bisporus*.

Table 3. Total protein content (PR) and ACE inhibitory activity (IC₅₀) of the different molecular weight fractions obtained from ExA and ExC. ^{a-f}Different letters denote significant differences ($P \leq 0.05$) between different samples for the same column.

Extract	Fraction	PR (mg/g fraction)	IC ₅₀ (mg fraction/mL)	IC ₅₀ /PR (mg protein/mL)
ExA	MW >10 kDa	391.93±2.28 ^a	0.45±0.01 ^c	0.18±0.03 ^{ab}
	10 > MW > 3 kDa	237.97±3.34 ^b	0.91±0.08 ^{bc}	0.22±0.02 ^a
	MW < 3 kDa	18.10±1.28 ^f	1.06±0.09 ^{bc}	0.02±0.00 ^c
ExC	MW >10 kDa	192.81±3.28 ^c	0.74±0.07 ^c	0.14±0.00 ^b
	10 > MW > 3 kDa	87.77±2.28 ^d	1.21±0.09 ^b	0.11±0.01 ^c
	MW < 3 kDa	35.49±1.09 ^e	1.63±0.15 ^a	0.06±0.00 ^c

The hypotensive activity of the obtained fractions was evaluated as their capacity to inhibit the angiotensin converting enzyme. However, since it was previously reported that certain peptides were responsible compounds a more detailed study was carried out on the two extracts including larger amounts of proteins (ExA

and ExC) by dividing them in 3 fractions of different molecular weights. The protein contents of fractions obtained from ExA were higher than those from ExC and within the fractions, lower molecular weight fractions contained less proteins, peptides and other N-containing compounds (Table 3). Fractions <10 kDa showed higher IC₅₀ than higher MW fractions ranging from 0.45 mg/mL for the MW>10 kDa fraction from ExA up to 1.63 mg/mL for the MW<3 kDa fraction from ExC. These values were lower than those reported in similar studies where other mushrooms such as *Hypsizygus marmoreus* (6.4 mg/mL) [11] or mycelia extracts from *Lactarius camphorates* (1.6 mg/mL) [12] were analyzed. A significant correlation between protein content and ACE inhibitory activity was found however, when IC₅₀ values were expressed taking into account the amount of proteins detected, fractions with lower molecular weight displayed the largest inhibitory capacity. Therefore, these results were in concordance with previous studies carried out on close related species such as *Lentinula polychrous* where small peptides were pointed as responsible compounds of their hypotensive properties [65].

Conclusions

This study showed that bioactive compounds can be differentially extracted from *Lentinula edodes* fruiting bodies or by-products using a sequential extraction method. Extracts obtained with cold water (ExA) contained high levels of water soluble β -D-glucans, chitoooligosaccharides and other carbohydrates that were pointed as potential responsible for the high HMGCR inhibitory activity showed in this extract. It also contained eritadenine with cholesterol lowering properties, lenthionine that can inhibit platelet aggregation, peptides with ACE inhibitory capacity and antioxidant phenols. Then, the residue after this extraction can be submitted to supercritical CO₂ extraction to obtain a fungal sterol-enriched fraction (ExB) with the ability of displacing cholesterol from dietary mixed micelles formed after digestion impairing its absorption. Afterwards, the remaining material can be submitted to hot water extraction yielding an extract (ExC) containing β -D-glucans with bile acid-binding capacities (able to interfere cholesterol absorption) and similar solubility than lentinan and a residue (ReC) including dietary fibres such as β -D-glucans and chitins that apparently, according to previous studies, modulate human microbiota reducing

the risk of CVDs. Therefore, many biologically active compounds can be separated within different fractions from the same batch of shiitake mushrooms if the described methods are sequentially applied. Then, the obtained fractions will contain higher concentrations of the bioactive compounds than the mushroom itself and they will be transformed into more bioaccessible forms (*i.e.* dietary fibers will be more easily fermented by colonic microbiota activating hypocholesterolemic mechanisms or absorbed by M cells in the intestine).

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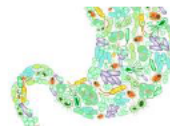
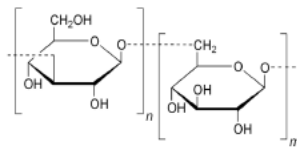
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Chapter 5

Testing the functional ingredient in a clinical trial



Preface

The fraction BGE was a β -D-glucan-enriched mixture (containing eritadenine and ergosterol) obtained as described in Chapter 3 that showed hypocholesterolemic activities *in vitro* and *in vivo* (Chapter 2). Therefore, in order to validate its biological activities, high amounts of the mixture were prepared and mixed with 3 commercial food matrices (asparagus and zucchini creams and gazpacho) to design a functional food for their administration to volunteers with non treated mild hypercholesterolemia in a randomized, placebo controlled, double-blind and parallel clinical trial.

In the work *Modulation of human intestinal microbiome in a clinical trial by consumption of a β -D-glucan-enriched extract obtained from *Lentinula edodes**, anthropomorphic parameters of all subjects were followed during the whole experimental time and a large diversity of biomarkers were monitorized before and after the treatment not only related to cholesterol homeostasis but also to their immune status (pro-inflammatory cytokines) since β -D-glucans were widely reported to show immunomodulatory capacities beside their hypocholesterolemic activity.

Moreover, since the BGE mixture contained a large amount of polysaccharides considered dietary fibres (chitins and β -D-glucans) and they might positive- or negatively modulate intestinal microbiome, the faecal microbiome of the subjects was studied before and after the intervention. Differences between the two timepoints and the two groups (BGE mixture and placebo) were evaluated using Next Generation Sequencing and bioinformatics tools. Microbiomal genera abundance and their correlations with the determined biomarkers were established to define the effect of the functional food consumed on human microbioma, immune system and risk of cardiovascular diseases.

Manuscript 1

Modulation of human intestinal microbiome by consumption of a β -D-glucan-enriched extract obtained from *Lentinula edodes*

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Abstract

A randomized, controlled, double-blind, and parallel clinical trial was carried out with subjects from 18 to 65 years old ($n = 52$) with not treated mild hypercholesterolemia. Volunteers consumed a specific mixture (10.4 g/day) obtained from shiitake mushrooms (*Lentinula edodes*) ensuring a 3.5 g/day of fungal β -D-glucans (BGE) or a placebo incorporated in 3 different commercial creams. This mixture showed hypocholesterolemic activities *in vitro* and in animal studies. After 8 weeks intervention, no significant differences in lipid- or cholesterol-related parameters were found compared to placebo subjects as well as before and after the BGE mixture administration. No inflammatory or immunomodulatory responses were noticed and no changes in IL-1 β , IL-6, TNF- α and oxLDL were recorded. However, consumption of the BGE mixture was safe and even managed to achieve the dietary fibre intake recommended as cardiovascular protective diet. Moreover, the BGE mixture modulated the colonic microbiome differently compared to placebo. Their microbiota community varied from before to after the intervention being some genera positive- or negatively correlated with some biomarkers related to cholesterol homeostasis. Nevertheless, the precise significance of this differential modulation was not fully elucidated and requires further studies.

Introduction

Cardiovascular diseases (CVD) are still leading the causes of death (particularly in developed areas) inducing disability and chronic diseases. However, they are disorders that can be reduced or prevented by adapting proper dietary habits and life styles. To help the positive influence of healthy diets, several ‘functional foods’ were developed in the last decades claiming effective lowering of cholesterol levels, hypotensive, antioxidant effects, etc. [1]. The most frequently consumed bioactive products are those including phytosterols (or derivatives) or β -D-glucans. However, there are some considerations to bear in mind when a subject is having a regular consumption of these products and particularly β -D-glucans e.g. they might not be as effective as expected and they might influence intestinal microbiome and immune system besides their hypocholesterolemic effect.

Although their mechanism of action is not completely understood, it is suggested that plant sterols impair absorption of dietary cholesterol [2]. The cholesterol-lowering effect of β -D-glucans might be related to the increase of viscosity in the intestine that stimulates the synthesis of bile acids from cholesterol to compensate for their fecal excretion after being scavenged within their complex structures and therefore, reduces the circulating LDL-cholesterol concentrations [3, 4]. However, if cholesterol is not acquired with the diet, liver activates the 3-hydroxy-methylglutaryl-coenzyme A reductase (HMGCR), stimulating its endogenous biosynthesis and then, some consumers do not manage to reduce their cholesterol levels by intaking of hypocholesterolemic food products. Moreover, a few studies correlated the effect of cholesterol lowering β -D-glucans with their potential as modulators of colonic microbiota [5, 6]. In fact, recent publications indicate that microbiome plays a key role in cholesterol regulation via several mechanisms but particularly by its conversion into coprostanol and further on to bile acids. The main taxa carrying out the first transformation were *Eubacterium* and *Bacteroides*. Afterwards, bile acids were modified (deconjugation, epimerization, oxidation, etc.) by a wide number of genera, modulating their solubility in physiological fluids to facilitate their reabsorption or to promote their secretion through feces. Strains of *Bifidobacterium* and *Lactobacillus* stimulated bile acid deconjugation and fecal

excretion promoting their hepatic biosynthesis from their main precursor: cholesterol. Then, a decrease of cholesterol levels in serum was noticed in hypercholesterolemic subjects [7].

Besides their effect on human microbiome, β -D-glucans were also pointed as immunomodulatory compounds directly interacting with specific receptors from the gut-associated lymphoid tissue (GALT) and the intestinal enterocytes triggering immune responses (releasing specific cytokines to activate both native and adaptive responses). β -D-Glucans also seemed to enhance GALT proper recognition of commensal microbiota and stimulate defensive responses against pathogens (i.e. IgA secretion into intestinal lumen) [8-10].

Edible mushrooms, and particularly shiitake mushrooms (*Lentinula edodes*), contain compounds that are known to have hypocholesterolemic properties [11]. Fungal sterols were acting as plant sterols by displacing cholesterol from dietary mixed micelles [2] and modulating the expression of cholesterol-related genes [12]. Shiitake β -D-glucans also scavenged bile acids according to *in vitro* digestion models [13]. Eritadenine, an S-adenosyl-L-homocysteine hydrolase (SAHH) inhibitor, could lower cholesterol levels by altering the hepatic phospholipid metabolism [14] and some water-soluble polysaccharides showed HMGCR inhibitory activities *in vitro* (Chapter 3, Manuscript 2). If the latter activity could also be effective *in vivo*, it might indicate that mushrooms could also inhibit the cholesterol biosynthetic pathway as drugs such as statins. Polysaccharides are large molecules that mostly undergo partial degradation during digestion and reach the colon. However, certain water-soluble polysaccharides might cross the intestinal barrier and enter the blood stream although their mechanism is not well understood (perhaps entering by M-cells or other means) [8].

When β -D-glucan-enriched extracts obtained from oyster mushrooms (*Pleurotus ostreatus*) were administrated to hypercholesterolemic mice together with lard (simulating an unhealthy diet), lowering of total cholesterol (TC) and LDL-cholesterol was noticed after 4-weeks administration [15]. Similar results were observed when mice following a high cholesterol diet were administrated shiitake polysaccharides [16] or water-soluble polysaccharide fractions [15]. An eritadenine-

containing fraction from shiitake mushroom was able to lower the atherogenic index (TC/HDL-C) in rat serum (Chapter 2, Manuscript 1). Only the ergosterol-enriched extracts seemed to be less effective in animal studies (Chapter 1, Manuscript 2) although they modulated the expression of genes related to cholesterol metabolism in mice following a similar profile as hypocholesterolemic drugs [12]. Moreover, a mixture of extracts obtained from *L. edodes* and formulated to contain all the previously mentioned compounds successfully lowered cholesterol levels in hypercholesterolemic mice after 5 weeks (Chapter 1, Manuscript 2). Therefore, in this work, that fungal mixture (BGE) was also tested in a clinical trial to investigate whether it could exert the noticed hypocholesterolemic effect in humans. Since the mixture contained a large β -D-glucan concentration, its effect on the cytokine profile and colonic microbiota of the volunteers was also evaluated.

Materials and Methods

Biological material and reagents

Powdered *Lentinula edodes* S. (Berkeley) fruiting bodies (particle size < 0.5 mm, moisture < 5 %) were obtained from Glucanfeed S.L. (La Rioja, Spain).

Maltodextrin (Sosa Ingredients, Moia, Spain) (5.2 g) was mixed with 1.6% food-grade brown dye (Sosa Ingredients, Moia, Spain) to prepare a placebo formula that resemble the visual appearance of the mushroom extract.

Preparation and analysis of the β -D-glucan extract from shiitake mushrooms

A specific β -D-glucan-enriched (BGE) mixture of two extracts obtained from *Lentinula edodes* fruiting bodies was prepared using a pilot scale solid/liquid extraction unit as described in Chapter 3, Manuscript 3. Briefly, the BGE was prepared by mixing different polysaccharides fractions such as a fraction (2.5%) containing water soluble β -D-glucans (named ExA in Chapter 4, Manuscript 1), a fraction (26%) extracted with hot water (98 °C), filtered through a multichannel ceramic membrane (Ceramem Corporation, Waltham, USA) and concentrated with a

spiral wound Nanomax50 membrane (Millipore, Bedford, USA) (called RF2 in Chapter 3, Manuscript 3) and the remaining fraction (71.5%) containing mainly chitins and insoluble β -D-glucans. The fractions were lyophilized, pooled together and stored at -20 °C until further use.

BGE proximate composition was quantified following the AOACs methods. Other constituents such as total carbohydrate, β -D-glucans, chitins, ergosterol, eritadenine and lenthionine contents were determined as described in Chapter 3, Manuscript 3).

Study subjects

Fifty two men and women from 18 to 65 years old were recruited according to the following inclusion criteria to be eligible for the study: BMI ≥ 18.5 and $< 30 \text{ kg/m}^2$, adequate cultural level and understanding for the clinical trial, signed informed consent, cardiovascular risk $< 10\%$ to 10 years measured by REGICOR, total cholesterol $\geq 200 \text{ mg/dL}$. They also included at least 1 factor in the following list: ≥ 45 years or women ≥ 55 years, family history of cardiovascular disease (CVD) in first degree male relative less than 55 years of age and less than 65 years in women; HDL cholesterol: men < 40 or women < 50 , triglycerides $\geq 150 \text{ mg/dL}$ and $< 200 \text{ mg/dL}$; LDL cholesterol $\geq 130 \text{ mg/dL}$ and $< 160 \text{ mg/dL}$; smoker; willingness to follow a healthy diet; hyperlipidemic control diet and a diet without stanols, sterols and yeast; social or familiar environment that prevents from accomplishing the dietary treatment. The exclusion criteria were as follows: individuals diagnosed with Diabetes Mellitus type 1 and 2 on pharmacological treatment, with dyslipidemia, with hypertension, with hypothyroidism; individuals > 60 years smokers with total cholesterol $> 200 \text{ mg/dL}$ or LDL $> 130 \text{ mg/dL}$; individuals allergic to shiitake mushrooms; individuals with chronic diseases (hepatic, kidney, etc.); individuals receiving pharmacological treatment that modifies the lipid profile (for example, statins, fibrates, diuretics, corticosteroids, ADOs); individuals who have participated in the last 6 months in a program or clinical trial to lose weight; smokers wanting to stop to smoke during the period that clinical trial lasts; individuals with mental illness; individuals consuming drugs to lose weight during 30 days before starting the study and pregnant or breastfeeding women.

All subjects gave their informed consent to take part in the study that was approved by the Scientific Research and Ethics Committee of the HULP (La Paz University Hospital) (Code 4813) in accordance with the ethical standards of the Declaration of Helsinki [17]. The study was registered at <http://clinicaltrials.gov> under the number NCT03550287.

Study design

The study was a randomized, controlled, double-blind, and parallel clinical trial lasting 8 weeks. Subjects ($n = 52$) were randomly assigned (maintaining the gender ratio of the sample) to one of two treatments involving a β -D-glucan-enriched mixture (Treatment Group (TG): 10.4 g/day BGE mixture ensuring a 3.5 g/day of β -D-glucans) or a placebo (Control Group (CG): an isocaloric dose of brown colored maltodextrin) incorporated in a commercial asparagus or zucchini creams or a Gazpacho (a traditional Spanish tomato puree). Neither the researchers nor the subjects knew to which treatment group the subjects were assigned to; the researchers were unblinded only at the end of the study.

Diet

All subjects were requested to follow a healthy diet, hyperlipidemic control diet and a diet without stanols, sterols and yeast. The diet of each subject was recorded during the week prior to the beginning and end of each intervention period. All food and beverages consumed inside and outside the home were recorded over three consecutive days (including one day of the weekend) [18]. Subjects were instructed to record the weight of the food consumed or, if this was not possible, to record household measurements (spoonfuls, cups, etc.). At each visit, all records were thoroughly reviewed by a nutritionist in the presence of the subject to ensure that the information collected was complete. The energy and nutritional content of the foods and beverages consumed were then calculated using DIAL software (Alce Ingeniería, Madrid, Spain).

Anthropometric variables and health variables

Anthropometric measurements were taken at the beginning and end of the intervention using standard techniques, adhering to international norms set out by the WHO (1976)[19]. All measurements were made by trained personnel in the morning with the subject barefoot and wearing only underwear. Height was determined using a height meter with an accuracy of 1 mm (range, 80–200 cm). Body weight was measured using a Tanita BC-420MA balance (Bio Lógica Tecnología Médica S.L, Barcelona, Spain). BMI was calculated using the following formula: $[\text{weight (kg)}/\text{height (m)}^2]$. Waist circumference (WC) was measured using a Seca 201 steel tape (Quirumed, Valencia, Spain). Information was collected on medical conditions and the consumption of medications. Blood pressure and heart rate were measured on the right arm using a Spot Vital Signs 420 automatic monitor (Welch Allyn, Madrid, Spain) (accuracy ± 5 mmHg).

Biochemical and haematological analysis

At the beginning and end of the intervention, blood samples were collected early in the morning at the Extraction Unit from La Paz University Hospital (HULP). Samples were kept at 4–6 °C until analysis, which was always performed within 48 h. Blood samples were obtained from fasting subjects at baseline and at 8 weeks at the HULP biochemical lab. The biochemical parameters evaluated were total cholesterol, HDL- and LDL-cholesterol, triglycerides concentrations as well as glucose, basal insuline, HbA1C, apolipoproteins A1 y B, creatinine, glomerular filtrate (CKD-EPI), ureate, transaminase (ASAT, ALAT), lipase, C-reactive protein, vitamin D (as calcidiol).

Other haematological data were also measured as leucocytes, erythrocytes, haemoglobin, hematocrit, VCM, HCM, CHCM, RDW, platelets and VPM, protrombin time and activity, INR, fibrinogen and a leucocyte differential was also included to distinguish between neutrophils, lymphocytes, monocytes, eosinophils, basophils since one previous publication indicated that shiitake consumption increased eosinophil levels together with gastrointestinal problems in a few subjects

[20]. All determination were measured with CORE Lab Systems (Siemens Healthcare®). Dimension Vista Intelligent Lab System in the HULP.

Inflammation and oxidative data

Subjects received the exact number of packages (in a box packaging) required for each intervention and they were asked to return all empty and non-empty packages. Compliance was measured at the middle of the intervention and at the end by comparing the number of packages provided and the number returned. A subject was considered compliant when he/she consumed $\geq 90\%$ of the packages provided. Adverse events were recorded on the middle and final visit of the intervention. An adverse event was defined as any unfavorable, unintended effect. All such events were recorded along with the symptoms involved (nausea, vomiting, diarrhea, constipation, etc.).

Statistical analysis

A sample size of 56 patients was calculated as the sample size that was necessary to provide 80% power (at $\alpha = 0.05$). Quantitative data are presented as the means \pm standard deviations (SD). Atypical data (i.e. lying more than two SDs from the mean) in asymmetric distributions were deemed to reflect true results; they were, therefore, not eliminated from the analysis. Qualitative data were presented as counts and percentages. The Kolmogorov–Smirnov test was used to determine whether the data were normally distributed. Levene’s test was used to assess the equality of variance. When the distribution of the results was normal, the Student t test was used to compare the mean values of the studied variables recorded for the two treatment groups. The Mann-Whitney U test was used when the distribution was not normal. Multiple comparisons were adjusted using the Bonferroni method. Two-sided tests were used, and a p -value < 0.05 was considered statistically significant. Statistical analyses were performed using SPSS v. 21.0 software (SPSS, Chicago, IL).

Fecal microbiota

Fecal depositions from all subjects were collected on a sterilized vessel at the beginning and end of the intervention and stored at -80°C . DNA extraction from

human faeces (0.2 g) was performed in duplicate using the Purelink™ Microbiome DNA Purification Kit from Invitrogen (Thermo Fisher Scientific, Madrid, Spain) following the manufacturer's instructions. Isolated DNA was quantified using a fluorimetric method with Quant-iT Picogreen Assay Kit (Thermo Fisher Scientific, Madrid, Spain). Then, obtained DNA (3 ng) was submitted to PCR (23 cycles) with Q5® Hot Start High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, USA) using the primers (50 nM) 5'-**ACACTGACGACATGGTTCTACA**GTGCCAGCMGCCGCGGTAA -3' and 5'-**TACGGTAGCAGAGACTTGGTCT**GGACTACHVGGGTWTCTAAT- 3' for amplification of V4 region of 16S ribosomal subunit. Afterwards, qPCR (12 cycles) was performed using the primers (400 nM): (5'-AATGATACGGCGACCACCGAGATCT**ACACTGACGACATGGTTCTACA** -3' and 5'-CAAGCAGAAGACGGCATACGAGAT-[10 nucleotides barcode]-**TACGGTAGCAGAGACTTGGTCT**-3') of the Access Array Barcode Library for Illumina Sequencers (Fluidigm Corporation, San Francisco, USA). The obtained amplicons were validated and quantified by Bioanalyzer 2100 (Agilent, Madrid, Spain) and an equimolecular pool was purified by agarose gel electrophoresis and titrated by quantitative PCR using the Kapa-SYBR FAST qPCR kit for LightCycler® 480 (Sigma-Aldrich, Madrid, Spain) and a reference standard for quantification. The amplicons pool was denatured prior to be seeded on a flowcell, in two runs, at a density of 7.5 and 9.5pM each, where clusters were formed and sequenced using a MiSeq Reagent Kit v3, in a 2x300 pair-end run on a MiSeq sequencer (Illumina, San Diego, USA).

Microbial community analysis

Raw reads were processed using the NG-Tax pipeline and SILVA database (Release 132) [21, 22]. The resulting OTU table in biom format was imported in R (v 3.5.0) for downstream analysis. Since, the samples were sequenced in duplicate, a correlation analysis was done using Pearson's correlation coefficient. Samples with an $R^2 < 0.90$ were removed. Overall, community level similarities/differences between replicates were visualized using principal coordinates analysis using UniFrac

distances. Since most samples were highly correlated samples form, replicate one was chosen for analysis reported in this study.

Phylogenetic diversity was calculated on rarified data (sample.size = 43425) using phyloseq (v 1.24.2) and picante (v1.6.2). Multivariate redundancy analysis was carried out using the vegan (v2.5.5) R package. For RDA, the OTU table was transformed using Hellinger transformation. To assess the role of biological parameters measured in our study, the *envfit* (permutations = 999) function was used. To assess the impact of treatment and control for inter-individual variation, the constrained RDA was performed (formula = ~ metadata\$Treatment_TimePoint + Condition(metadata\$SubjectID)). The significance of difference was tested using the *permutest*, with 999 permutations. For specific analysis of the treatment group, the data was filtered to include only TG samples. RDA analysis was performed on Hellinger transformed OTU table and using the formula mentioned above. The genus level relative abundance data was used as variables in *envfit* (permutations = 999) analysis to identify genera responsive to the treatment. This analysis included only those genera that had a minimum relative abundance of 0.001 in at least 20% of the samples in TG group. All visualizations were done using ggplot2 (v3.1.1) and ggpubr (v0.2) package.

Results and discussion

Table 1. Composition of the β -D-glucan-enriched (BGE) mixture obtained from shiitake mushrooms

Compound	(g/100g extract dw)
Proteins	21.3 \pm 0.3
Total carbohydrates	47.6 \pm 2.1
β -D-glucans	33.5 \pm 2.3
Chitins	6.8 \pm 0.3
Fat	5.7 \pm 0.3
Ergosterol	0.23 \pm 0.01
Eritadenine	0.27 \pm 0.01
Ash	5.7 \pm 0.1

The BGE preparation obtained from *Lentinula edodes*, besides dietary fibers i.e. β -D-glucans and chitins (Table 1), contained other compounds with potential

hypocholesterolemic activities such as water-soluble polysaccharides (α - and β -D-glucans and fucmannogalactans) ergosterol and eritadenine [11, 23].

However, the lipid content of the BGE mixture was low, thus, ergosterol was present possibly in insufficient amounts to be effective since, when it was administrated in concentrations up to 0.45% of the diet, no lowering of serum cholesterol was noticed in mice (Chapter 1, Manuscript 2). Similarly, eritadenine intake was lower than 10 mg/kg/day given to mice in experimental assays (Chapter 2, Manuscript 1), therefore, the major hypocholesterolemic compounds present in the extracts mixture were soluble and particularly insoluble polysaccharides. They were supplemented to commercial food matrices and incorporated into a CVD protecting diet.

Study population and baseline characteristics

Fifty two hypercholesterolemic subjects without pharmacological treatment (38 women (74.5%), 14 men (25.5%)) and with a mean age of 50.8 ± 10 years old finished the study (Figure 1).

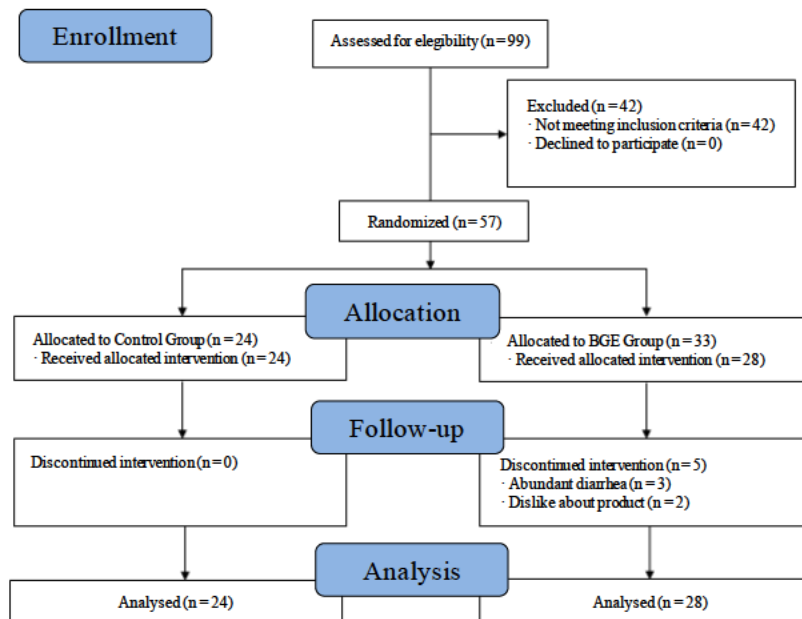


Figure 1. Flow diagram describing the clinical trial

Fifty two hypercholesterolemic subjects without pharmacological treatment (38 women (74.5%), 14 men (25.5%)) and with a mean age of 50.8 ± 10 years old finished the study (Figure 1).

Table 2. Anthropometric characteristics of the study population according to the assigned treatments with placebo (CG) or BGE mixture (TG) (mean \pm SD). BGE: β -D-glucan-enriched; BMI: Body Mass Index; WC: Waist circumference; SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure; HR: Heart Rate; * $P < 0.05$, ** $P < 0.01$ significantly different compared to baseline; NS, not significant.

		CG	TG	<i>P</i>	
Weight (kg)	Baseline	67.26±11.64	72.23±13.36	NS	
	Final	66.25±11.11	71.5±13.55*		
	Change	-1.01±3.21	-0.73±1.77	NS	
BMI (kg/m²)	Baseline	24.54±3.09	26.01±3	NS	
	Final	24.43±2.84	25.73±3.01*		
	Change	-0.11±1.58	-0.28±0.65	NS	
WC (cm)	Baseline	87±8.86	90.43±11.11	NS	
	Final	87±8.71	90.34±11.69		
	Change	-5.77±21.28	-14.85±34.07	NS	
Blood Pressure	SBP (mmHg)	Baseline	106.63±12.68	113.68±14.11	NS
		Final	115±13.02**	121.18±16.49**	
		Change	4.53±10.36	7.54±12.99	NS
	DBP (mmHg)	Baseline	73.08±12.01	75.64±10.18	NS
		Final	73.42±10.13	76.36±10.01	
		Change	2.6±7.4	0.04±8.9	NS

The analyses were carried out with 28 participants in TG group (19 women, 9 men) and 24 in CG group (19 women, 5 men). The mean BMI was 25.3 ± 3.1 kg/m² (overweight) and the waist circumference (WC) was of 88.9 ± 10.2 cm (85.8 ± 9.1 cm in woman and 97.1 ± 8.3 cm in men). The mean blood pressure was normal ($110.4 \pm 123.8/74.5 \pm 11.0$ mmHg).

Compliance and adverse events

All subjects ingested $\geq 90\%$ of the product provided. No significant differences were observed in the number of packages consumed between the different treatments. The main adverse events resulting from the intake of the BGE mixture were swelling ($n=4$), heartburn ($n=3$) and flatulence ($n=2$), while in subjects of the CG were swelling ($n=3$), flatulence ($n=2$) and diarrhea ($n=2$). These results were in line with another clinical study where the administration of a soluble beta-glucan preparation (containing lentinan) from shiitake mycelium induced similar adverse events (in number, nature and severity) to placebo group [24].

Effect of the BGE mixture on general parameters of the population

After 8 weeks, the subjects from TG showed a significant reduction of weight and BMI compared to the beginning of the study (Table 2). However, this reduction was not significant when it was compared with the CG. These results were similar to others studies using other mushrooms from the same order (Agaricales) where weight or BMI did not significantly change with the intake of soup containing 30 g dried oyster mushrooms (*Pleurotus ostreatus*) consumed over the study period of 21 days [25]. One-year randomized clinical trial examined the effect of substituting red meat by white button mushroom (*Agaricus bisporus*) compared to a standard diet on weight loss in obese adults. At the end of the trial, participants on the mushroom diet lost more weight, achieved lower BMI and waist circumference compared to the beginning of the study. However, these changes were also not significantly different when they were compared to the standard diet group [26]. On the other hand, in the present study, both intervention groups increase the systolic blood pressure from the beginning to the end of the intervention ($P<0.01$) with no significant differences between treatment groups and without clinical relevance. These observations disagreed with previous reports where diabetic subjects consuming oyster mushrooms significantly reduced their systolic and diastolic blood pressure [27]. The different mushroom species tested or volunteers pathology might be the reasons for the different effects. In the subjects of this study (with mild and not treated

hypercholesterolemia), weight, BMI, WC, and blood pressure showed no significant differences between intervention groups.

Effect of the BGE mixture on diet and cholesterol-related parameters

After the intervention ended, the CG showed better monitoring of healthy feeding and hyperlipemia control guidelines than the TG (Table 3).

Table 3. Dietetic parameters of the study population according to the assigned treatments with placebo (CG) or BGE mixture (TG) (mean \pm SD). BGE: β -D-glucan-enriched; SFA: Saturated Fatty Acids; MUFA: Monounsaturated Fatty Acids; PUFA: Polyunsaturated Fatty Acids; * $P < 0.05$, ** $P < 0.01$ significantly different compared to baseline; NS: Not significant.

		CG	TG	<i>P</i>
Energy (kcal)	Baseline	1975.2 \pm 537	1903 \pm 593.5	NS
	Final	1648.8 \pm 481.4**	1779.6 \pm 399.6	
	Change	-338.9 \pm 634.6	-48.4 \pm 406.7	0.037
Proteins (g)	Baseline	78.1 \pm 21.9	82.9 \pm 27	NS
	Final	67.8 \pm 18.1*	76 \pm 19.1	
	Change	-10.2 \pm 22.6	-4.3 \pm 20.7	NS
Carbohydrates (g)	Baseline	200.5 \pm 62.7	186.6 \pm 65.9	NS
	Final	164.8 \pm 55.7*	165.5 \pm 35.9	
	Change	-37.1 \pm 72.5	-15.3 \pm 48.1	NS
Lipids (g)	Baseline	86.1 \pm 25.5	81.9 \pm 30.9	NS
	Final	70.4 \pm 24**	81.1 \pm 30.4	
	Change	-16.5 \pm 34	3.1 \pm 31.1	0.028
SFA (g)	Baseline	27 \pm 10.4	22.2 \pm 8.7	NS
	Final	19.4 \pm 8.7**	23.8 \pm 17.1	
	Change	-7.8 \pm 12.8	2.7 \pm 15.7	0.022
MUFA (g)	Baseline	38.3 \pm 12.2	39 \pm 17	NS
	Final	30.3 \pm 11.2**	34.8 \pm 15.1	
	Change	-8.3 \pm 16.8	-2.4 \pm 16.6	NS
PUFA (g)	Baseline	12.2 \pm 3.6	12.4 \pm 5.2	NS
	Final	9.5 \pm 4.1*	10.9 \pm 4.5	
	Change	-2.7 \pm 5.1	-0.9 \pm 5.1	NS
Cholesterol (g)	Baseline	308.9 \pm 160.2	273.2 \pm 158.7	NS
	Final	266.8 \pm 143.2	273.2 \pm 158.7	
	Change	-38.4 \pm 234.7	21.3 \pm 129.2	NS
Fiber (g)	Baseline	19.1 \pm 6.7	22.1 \pm 7.9	NS
	Final	16.3 \pm 4.8	25.2 \pm 7*	
	Change	-1.8 \pm 8.0	3.1 \pm 7.7	0.015

The CG significantly reduced energy intake (-338.9 ± 634.6 vs. -48.4 ± 406.7 kcal; $P < 0.05$), total lipid consumption (-16.5 ± 34.0 vs. 3.1 ± 31.1 g; $P < 0.05$) and SFA (-7.8 ± 12.8 vs. 2.7 ± 15.7 g; $P < 0.05$) of the diet compared to the TG. Moreover, the CG also significantly reduced the levels of proteins, carbohydrates, MUFAs and PUFAs compared to the start of the intervention but these changes were not significant when compared to TG. Cholesterol consumption was similar in both intervention groups showing no change throughout the study.

However, the changes observed in diet among intervention groups did not affect the lipid profile of study participants who did not show significant changes for any of the variables related to lipid metabolism, including LDL (Table 4). Although the BGE mixture contained 3.5 g/day of fungal β -D-glucans and regular consumption of 3 g/day of oat and barley β -D-glucans was indicated as the effective concentration to notice a significant reduction of LDL cholesterol in serum [28, 29], no effect on total cholesterol or lipoproteins was observed. These results were in line with some clinical trials where subjects were administrated fruiting bodies or extracts obtained from other mushroom species. Schneider et al. (2011) [25] did not notice significant changes in LDL and HDL-cholesterol levels after eating 30 g/day of dried oyster mushrooms over a period of 21 days compared to placebo group. In a double-blinded, placebo-controlled, cross-over intervention study of 4 weeks the supplementation with reishi (or lingzhi) mushroom (*Ganoderma lucidum*) did not significantly change the plasma lipids even when a small decrease was observed in total and LDL-cholesterol [30]. The intravenously administration of a shiitake extract (containing lentinan) to healthy elderly subjects also failed to modify TC, LDL, HDL levels during the 6 weeks intervention and compared to placebo subjects [24]. However, Khatun et al. (2007)[27] reported reduction of TC and TG with no effect on HDL during the periods where diabetic subjects were consuming oyster mushrooms in a 24 days experiment (7-days mushroom consumption, 7-days no consumption and 7-days restarting consumption). Thus, apparently the hypocholesterolemic effect was only noticed in diabetic patients and not in healthy individuals or subjects with mild hyperlipemia as those included in the present study.

On the other hand, the different linking patterns between cereal ((1→3),(1→4)-β glucosidic bounds) and fungal (1→3),(1→6)-β-D-glucans might result in different molecular folding generating different intestinal viscosity or affinity to scavenge bile acids [31, 32]. According to *in vitro* tests, fungal β-D-glucans showed similar bile acid binding capacities than a mixture of cereal β-D-glucans with cholesterol lowering properties [13]. Therefore, the insignificant reduction of cholesterol levels in serum by fungal β-D-glucans might be related to the possible different intestinal viscosity generated. Viscosity in the intestine is also modulated by concentration, molecular weight and β-D-glucans solubility [33]. The BGE mixture contained both soluble and insoluble β-D-glucans plus chitins.

Table 4. Parameters of lipid metabolism of the study population according to the assigned treatments with placebo (CG) or BGE mixture (TG) (mean ± SD). NS: non significant differences.

		CG	TG	P
Cholesterol (mg/dL)	Baseline	237.46±31.85	240.04±27.9	NS
	Final	238.13±39.83	240.71±41.03	
	Change	0.67±26.41	0.68±27.99	NS
HDL (mg/dL)	Baseline	61.33±14.82	57.07±13.08	NS
	Final	60.04±14.09	55.96±10.58	
	Change	-1.29±7.82	-1.11±7.61	NS
LDL (mg/dL)	Baseline	157.5±30.99	158.54±26.64	NS
	Final	158.04±35.71	161.71±37.59	
	Change	0.54±28.35	3.18±25.03	NS
Triglycerides (mg/dL)	Baseline	98±47.11	133.36±101.95	NS
	Final	100.83±44.15	123.57±66.36	
	Change	2.83±42.88	-9.79±65.47	NS

Wise to mention was the fact that, although the TG showed a less healthy dietary pattern throughout the study, no significant variations in plasma levels of total cholesterol were observed. This could be due to the increase in total fiber consumption of the TG diet with respect to the control group (-2.8±8.0 vs. 3.1±7.7 g). The noticed increase in fiber consumption was mainly due to their supplementation with the BGE mixture. In fact, only the TG consumers achieved the recommendation of dietary fiber for the general population (>25 g/d) [34]. EFSA and similar

authorities recognize that diets with fiber levels higher than 25 g per day reduce risk of coronary heart diseases and type 2 diabetes and improve weight maintenance, therefore, consumption of the BGE mixture was favorable for that group.

Effect of the BGE mixture on LDL oxidation

Increased levels of circulating oxLDL are involved in the pathogenesis of atherosclerosis and they are associated with clinical atherosclerotic cardiovascular disease events [35]. However, no significant changes were observed in oxLDL concentrations after BGE mixture administration, indicating that the dose used in the study did not influence its levels (Table 5). Other studies using oyster mushrooms observed significant reduction of oxLDL concentrations from the beginning to the end of the mushroom diet consumption while no change was observed in the placebo group. Nevertheless, differences between groups were not significant suggesting that other components of the diet might be the responsible for those differences [25].

Effect of the BGE mixture on cytokines levels

After 8 weeks of intervention, no significant changes were observed in the determined cytokines concentrations between the treatment groups (Table 5). These results were in concordance with a double blind, crossover, and placebo-controlled trial carried out in 42 healthy elderly subjects (> 65 years). All participants consumed either 2.5 mg/day of an extract containing water-soluble β -D-glucans from *L. edodes* mycelium or placebo for 6 weeks; then after a washout period of 4 weeks, the alternate supplementation was given for 6 weeks. At the end of the study, factors of the immune response as immunoglobulins, complement proteins or cytokines (including TNF- α) were not altered by the β -D-glucan consumption [24]. These results differed from those reported by Dai et al. (2015)[36] where the pattern of cytokines secreted before and after shiitake mushrooms consumption was significantly different. Apparently, after 4 weeks, the consumption of either 5 or 10 g/day of whole fruiting body by healthy adults (21 – 41 years) resulted in an increased TNF- α and IL-1 α levels but no changes in IL-6 or IL-1 β concentrations. The secretion of the anti-inflammatory cytokines IL-4 and IL-10 was stimulated in large quantities

after the mushrooms consumption but the pro-inflammatory TNF- α levels were also increased. However, the lack of a placebo group in the study made impossible to conclude whether these changes were due to the mushroom administration and to draw further comparison with the present study despite the similarities in the age range.

Table 5. Cytokines and oxLDL of the study population according to the assigned treatments with placebo (CG) or BGE mixture (TG) (mean \pm SD). NS: non significant.

		CG	TG	P
oxLDL (ng/mL)	Baseline	44.14 \pm 37.91	73.86 \pm 153.1	NS
	Final	37.14 \pm 28.63	76.1 \pm 113.17	
	Change	7.01 \pm 46.67	0.57 \pm 204.68	NS
IL1- β (pg/mL)	Baseline	0.19 \pm 0.08	0.22 \pm 0.13	NS
	Final	0.2 \pm 0.12	0.24 \pm 0.12	
	Change	0 \pm 0.15	-0.03 \pm 0.14	NS
IL-6 (pg/mL)	Baseline	3.98 \pm 12.56	0.7 \pm 1.75	NS
	Final	2.38 \pm 7.58	2.64 \pm 11.28	
	Change	1.77 \pm 15.52	-1.88 \pm 11.59	NS
TNF- α (pg/mL)	Baseline	1.92 \pm 0.98	1.71 \pm 0.88	NS
	Final	1.94 \pm 1.03	1.76 \pm 0.9	
	Change	0.05 \pm 1.57	-0.08 \pm 1.16	NS

Moreover, animal studies indicated that the immune-regulatory functions of edible mushrooms (particularly *A. bisporus*) *in vivo* are difficult to detect since they can only be noticed after a challenge (for instance with dextran sodium sulfate, DSS). Then, they enhanced certain transient protection from colonic injury and induce a modest TNF- α secretion locally in the colon [37].

Effect of the BGE mixture on colonic microbiome

Fecal samples from all the subjects from TG and CG groups were used for the microbiota profiling. The 16S rRNA gene (variable region V4) was PCR amplified in duplicates for each sample and sequenced as technical replicates. In total, 15,472,993 reads were obtained with a minimum of 10,888 and a maximum of 170,682 reads per sample. These samples comprised of a total of 1434 OTUs before

quality filtering (see methods). The correlation between sequencing replicates for each sample was calculated and 10 samples showed a Pearson's correlation coefficient <0.90 (Supplementary table 1), therefore, they were excluded. Ordination analysis using UniFrac distances was used to visually identify similarities in community composition between replicates (Supplementary figure 1). Based on high correlation between replicates and UniFrac distances, one of the replicates was chosen for further analysis. Thus, the analysis was done on samples obtained before and after the intervention from 17 subjects from CG and 25 subjects from TG.

The phylogenetic diversity before and after intervention with BGE was measured and no statistically significant difference was noticed (Wilcoxon-test, $p > 0.05$) in CG as well as in the TG (Figure 2). Inter individual differences were observed in response to the intervention in both groups.

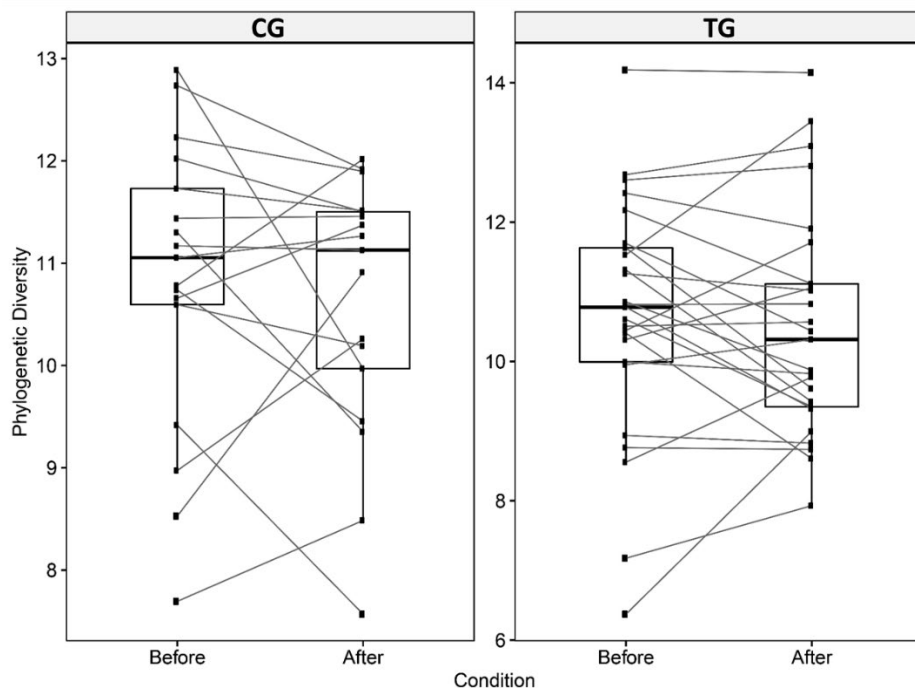


Figure 2. Phylogenetic diversity before and after intervention between the CG group (placebo) and TG (BFG mixture).

Moreover, associations between microbiota composition and several metadata features related to dietary intake, blood markers and cytokines were tested using redundancy analysis followed by *envfit* analysis (Supplementary Table 2). Among the categorical variables, subject identity showed the highest effect on observed microbiota variation ($R^2 = 0.94$, $p = 0.001$) in concordance with the observed interindividual differences in PD. In addition, among the continuous variables, dietary fiber intake ($R^2 = 0.23$, $p = 0.001$) and cholesterol concentration ($R^2 = 0.197$, $p = 0.001$) showed highest effect on microbiota variation. When subject identity was used as a conditioning covariate for assessing the effect of treatment in both TG and CG, a significant effect of treatment and time point on the microbiota community was observed (Figure 3A, permutation test, $P < 0.005$).

These observations indicated that the changes in the general microbiota during the 8 weeks of the study within the TG and CG groups were possibly due to their differences in fiber intake and cholesterol levels. To specifically assess differences in microbiota before and after intaking of the BGE mixture (TG), only TG samples were used in the RDA analysis using metadata variables and subset of genera (filtered using minimum relative abundance of 0.005 in at least 10/50 samples) as co-variates (Figure 3B). The analysis revealed that samples at baseline were associated with the presence of several *Ruminococcaceae* groups. Results also indicated that some of the key biomarkers of the cholesterol metabolism such as LDL, Apolipoprotein B and total cholesterol levels together with other unexpected variables (erythrocyte and hematocrit levels) were correlated ($p < 0.01$) to differences in microbiota community after intervention with BGE. In addition, the most responsive genera to intervention were *Oscillibacter*, *Lachnoclostridium*, *Bacteroides* and *Roseburia* ($p < 0.01$). The latter two genera, particularly *Bacteroides*, were previously reported as microorganisms involved in the metabolism of bile acids that regulates host cholesterol levels [7, 38].

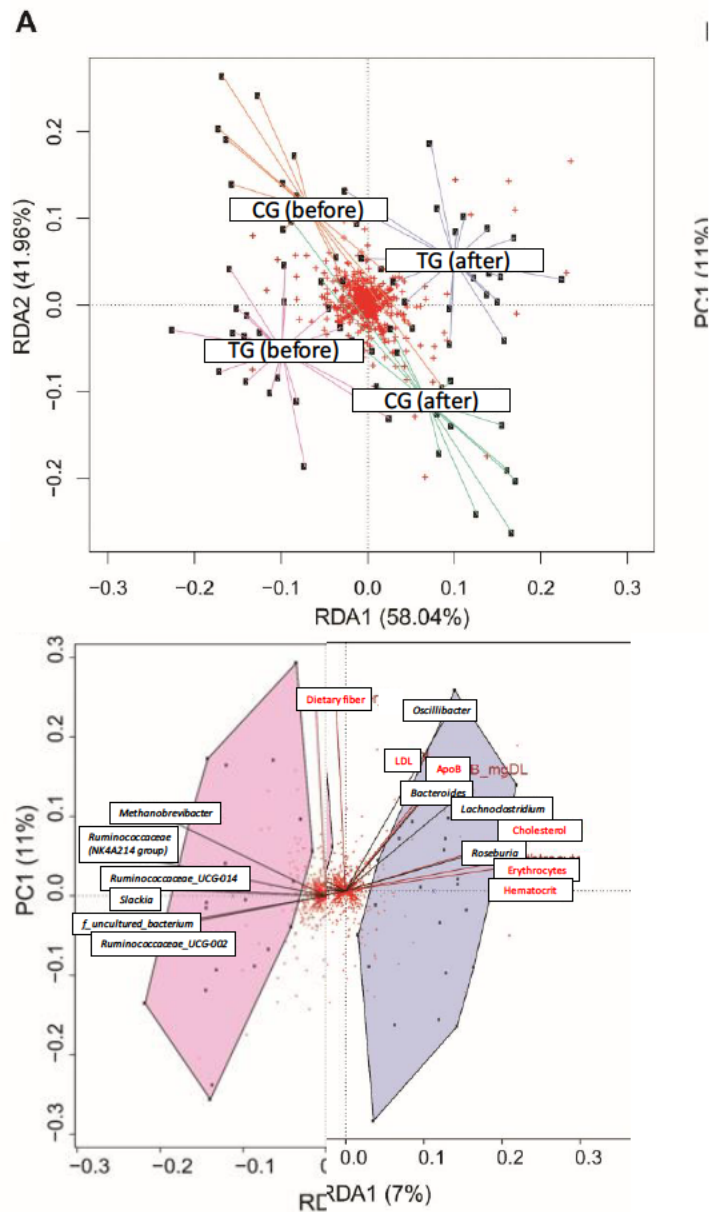


Figure 3. Redundancy analysis (RDA) of metadata co-variables and genus level abundances of prevalent taxa. A) RDA constrained for treatment time point and controlled for between subject variability. B) RDA with fitting of significant parameters and genera responsive to intervention (left cluster: TG before intervention, Right cluster: TG after intervention).

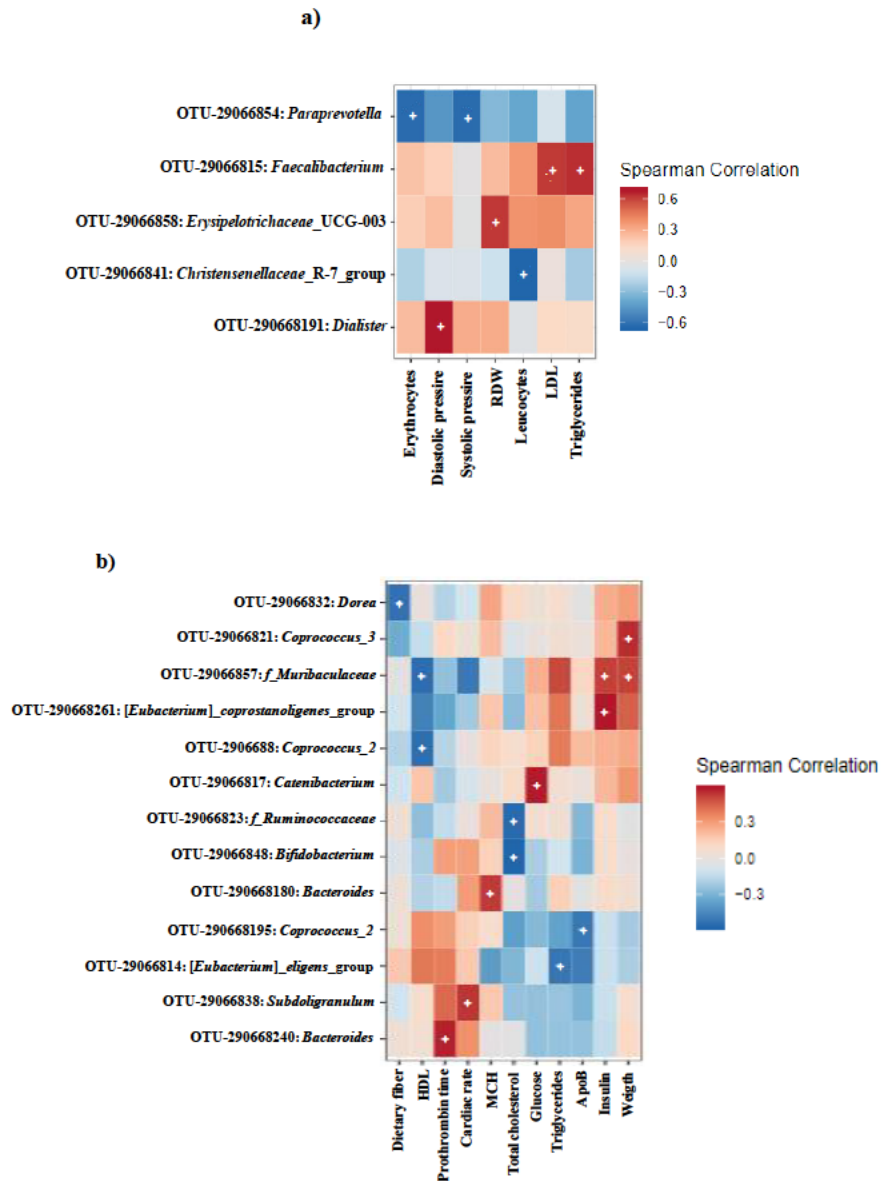


Figure 4. Association analysis between microbiota and biomarkers. A) ASVs showing significant correlation with biomarkers in CG. B) ASVs showing significant correlation with biomarkers in TG. Significant associations ($adj. p$ threshold > 0.05) are denoted by “+” sign. RDW: Red blood cell distribution width; MCH: Mean corpuscular hemoglobin.

Moreover, association studies were carried out between taxa abundances and the corresponding host related biological parameters for blood and immunological biomarkers (Figure 4). In the CG, strong correlations were observed with hematological data i.e. negative association was noticed between *Christensenellaceae* and leucocytes and *Paraprevotella* and erythrocyte counts, etc. but also with systolic and diastolic blood pressures. The latter observations might lack significance being artefacts since the correlation was indicated for only one of the pressures although previous studies linked *Paraprevotella* genus with profiles with high CVD risk [39, 40].

However, *Faecalibacterium* showed a positive association with LDL and triglycerides levels complementing the results from Wang et al. (2016) [5] that noticed a positive correlation of this genus with BMI and a negative one with HDL. In the TG, *Dorea* was inversely correlated with dietary fiber intake, being in concordance with previous clinical trials including β -D-glucan or fiber administration that noticed a significant reduction of *Dorea* after treatment [5, 41]. One *Coprococcus* OTU was positively correlated with weight, while other was inversely correlated with HDL ($\rho = -0.53$, $p = 0.03$). *Coprococcus* spp. were abundant in obese children in previous studies [42] and in rats fed high cholesterol diets [43]. *Muribaculaceae* genus was also inversely correlated with HDL ($\rho = -0.53$, $p = 0.03$) and positively correlated with weight and insulin. The latter metabolite was also positively associated with an OTU named *Eubacterium coprostanoligenes* group. Recently, *Eubacterium halli* demonstrated an improvement on insulin sensitivity after oral administration to obese and diabetic mice [44]. Other related group, *Eubacterium eligens* was inversely related with triglycerides levels ($\rho = -0.51$, $p = 0.048070169$), on contrary that observed with a relative close specie *Eubacterium rectale* that was positively correlated with triglycerides [45]. Nevertheless, most of the cholesterol-reducing bacteria isolated and characterized are members of the genus *Eubacterium* [7]. Previous works stated that *Catenibacterium* abundance was significantly increased after a high fat and high sugar diet [46], being in concordance with the positive correlation with observed glucose levels. Total cholesterol was inversely correlated with an OTU from *Ruminococcaceae* family ($\rho = -0.55$, $p = 0.02$) and *Bifidobacterium* genus ($\rho = -0.57$, $p = 0.01$). Besides some contradictory results

regarding *Ruminococcaceae* and lipidic profiles [47] observed lower *Ruminococcaceae* abundance in high-fat-fed mice while Sun et al., 2019 [48] noticed higher abundances in high-fat-fed hamsters), Liu et al., 2015 [49] detected positive correlations between *Ruminococcaceae* family and total cholesterol, LDL and LDL/HDL ratio in Japanese quail cecal microbiota. Higher consensus was achieved about the *Bifidobacterium* negative correlation with cholesterol, since the ability of this bacteria to lower cholesterol levels was largely reported [7, 50, 51].

Conclusion

Although the BGE mixture showed hypocholesterolemic effects in animal studies, it was not able to significantly lower the cholesterol levels compared to placebo in human trials. It contained a large fungal β -D-glucans concentration with reported immunomodulatory properties but they did not significantly modify the cytokine profile nor the oxLDL values of the subjects. However, consumption of the BGE mixture was safe and even might be positive because even having a less healthy diet (compared to placebo subjects) consumers managed to avoid increase of their cholesterol levels and achieving the dietary fibre intake recommended by authorities as healthy and cardiovascular protective diet. Moreover, the BGE mixture administration during 8 weeks modulated the colonic microbiome differently compared to placebo. In subjects administrated the mixture, the differences in microbiota community from before to after the intervention correlated with some metabolites related to cholesterol homeostasis being a few genera particularly responsive to the intervention. Within the microbiome of the BGE treated subjects, despite other groups and cholesterol-related metabolites, *Ruminococcaceae* family and *Bifidobacterium* genus were inversely associated to cholesterol levels but no correlation was found with any immunological parameter. Therefore, results from this study suggested a slight relation between cholesterol metabolism, microbiome and administration of the BGE mixture but still remains inconclusive, perhaps because the period of time or dose selected for the intervention was insufficient to achieve effective cholesterol reduction. It might be that the extra fiber intake recorded was starting to induce the microbiome modulation toward an improved cholesterolemic

status but it was ended before differences were significant. If this was the case, fungal β -D-glucans might act less effective than cereal β -D-glucans. However, longer period clinical trials would be necessary to clarify this proposed possibility.

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Supplementary material

Supplementary Table 1. Pearson's correlation coefficients between replicates for each sample

Sample code	Correlation coefficient
V1-1	0.983898155
V1-2	0.838465937
V1-3	0.564884059
V1-4	0.988705234
V1-6	0.982568637
V1-7	0.994085291
V1-8	0.978686626
V1-9	0.953335652
V1-10	0.99347866
V1-12	0.958260388
V1-13	0.890204434
V1-14	0.908235003
V1-15	0.961995533
V1-16	0.988959089
V1-17	0.991575206
V1-18	0.989416706
V1-19	0.947760361
V1-21	0.835265634
V1-22	0.995220119
V1-23	0.990251555
V1-24	0.921202948
V1-26	0.994125178
V1-27	0.961655984
V1-28	0.932922265
V1-29	0.994138143
V1-30	0.988925496
V1-31	0.987745227
V1-32	0.992673162
V1-33	0.924787687
V1-34	0.973443255
V1-35	0.908313951
V1-36	0.983387503
V1-37	0.967009892
V1-38	0.991614014
V1-39	0.86363053
V1-40	0.879386348
V1-41	0.984676206
V1-42	0.739537116
V1-43	0.996050983
V1-44	0.983294189
V1-45	0.97288901
V1-46	0.974518107
V1-47	0.985314536
V1-48	0.947390846

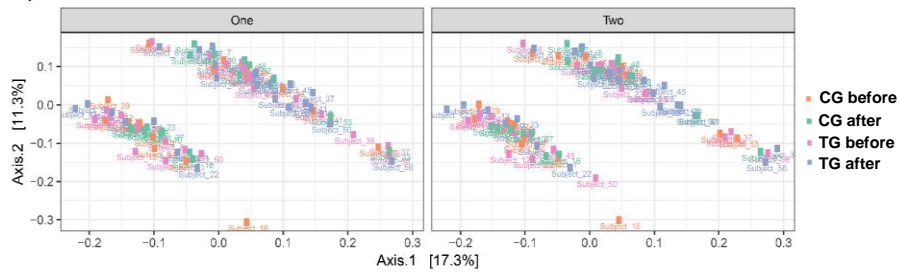
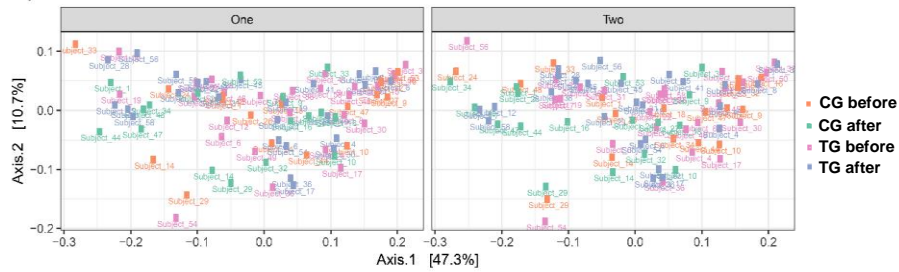
V1-49	0.935526027
V1-50	0.994177018
V1-52	0.987899281
V1-53	0.921732186
V1-54	0.994682636
V1-56	0.997672712
V1-58	0.989585801
V2-1	0.977986883
V2-2	0.987071076
V2-3	0.971725549
V2-4	0.981389677
V2-6	0.993226232
V2-7	0.990232176
V2-8	0.994012982
V2-9	0.985571402
V2-10	0.978863064
V2-12	0.99401274
V2-13	0.993654367
V2-14	0.985994059
V2-15	0.937068324
V2-16	0.928753235
V2-17	0.985370541
V2-18	0.973079956
V2-19	0.993395315
V2-21	0.937372489
V2-22	0.983657195
V2-23	0.965422324
V2-24	0.944015858
V2-26	0.954410114
V2-27	0.985692741
V2-28	0.963566706
V2-29	0.981684073
V2-30	0.96715792
V2-31	0.964522561
V2-32	0.980196603
V2-33	0.982150219
V2-34	0.960152894
V2-35	0.779913849
V2-36	0.994238784
V2-37	0.965867769
V2-38	0.997009324
V2-39	0.939263425
V2-40	0.98262872
V2-41	0.997393619
V2-42	0.871928673
V2-43	0.695606113
V2-44	0.958597884
V2-45	0.995998792
V2-46	0.979364892
V2-47	0.993559225
V2-48	0.980246723

V2-49	0.96960666
V2-50	0.96507458
V2-52	0.993885888
V2-53	0.975507183
V2-54	0.937681258
V2-56	0.947720791
V2-58	0.994509364

Supplementary Table 2. Results from *envfit* (permutations = 999) function for continuous and categoric variables. AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular haemoglobin concentration, RCW: Red blood cell distribution width, INR: International normalized ratio, HbA1C: Hemoglobin A1C, oxLDL: oxidized LDL.

	PC1	PC2	R2	PR (>R)	
Age	0.45037	-0.89284	0.0263	0.358	
Dietary fiber	0.02049	-0.99979	0.2252	0.001	***
Cholesterol	0.9996	-0.02842	0.1971	0.001	***
HDL	0.91350	0.40684	0.0025	0.905	
LDL	0.93205	-0.36233	0.1139	0.006	**
Triglycerides	0.27387	-0.96177	0.0012	0.960	
ApoA1	0.99760	0.06924	0.0178	0.489	
ApoB	0.87162	-0.49019	0.1210	0.009	**
Creatinin	0.89016	0.45565	0.001	0.894	
Glomerular filtrate	0.31364	-0.94954	0.0112	0.619	
Urate	0.97904	0.20366	0.0481	0.113	
AST	0.70752	-0.70669	0.0187	0.480	
ALAT	0.57194	0.82030	0.0215	0.434	
Lipase	0.99987	0.01631	0.0085	0.614	
C-reactive protein	0.04831	0.99883	0.0076	0.702	
Vitamin D	0.66121	-0.7502	0.0016	0.930	
Systolic pressure	0.46300	-0.88636	0.0761	0.043	*
Diastolic pressure	0.99438	-0.10590	0.0523	0.123	
Cardiac rate	0.46562	0.88498	0.0528	0.121	
Bile acids	0.19705	-0.98039	0.1019	0.028	*
Leucocytes	0.50786	0.86144	0.1001	0.012	*
Erithrocytes	0.99763	-0.06878	0.1511	0.001	***
Hemoglobin	0.99779	0.06645	0.1784	0.001	***
Hematocrit	0.99779	0.06645	0.1784	0.001	***
MCV	-0.29310	0.95608	0.0192	0.443	
MCH	-0.17895	0.98386	0.0723	0.040	*
MCHC	-0.09548	0.99543	0.0800	0.026	*
RDW	0.99954	0.03043	0.1018	0.015	*
Platelets	0.66555	-0.74636	0.0054	0.816	
Prothrombin time	-0.49419	0.86935	0.0279	0.356	
Prothrombin activity	0.56829	-0.82283	0.0213	0.473	
INR	0.65085	0.75921	0.0145	0.563	
Fibrinogen	0.98778	0.15586	0.0030	0.874	
Glucose	0.89117	-0.45366	0.0107	0.610	
Insulin	0.28939	0.95721	0.0158	0.527	
NGSP HbA1c	0.34808	-0.93746	0.0335	0.246	
IFCC HbA1c	0.34809	-0.93746	0.0336	0.244	
Weight	0.52310	0.85227	0.0548	0.094	
Waist circumference	0.43060	0.90254	0.0370	0.220	
IL-1 β	-0.97026	0.24208	0.0490	0.131	
IL-6	-0.76706	-0.64157	0.0077	0.577	
TNF- α	-0.21150	-0.97738	0.0063	0.784	
oxLDL	-0.99774	0.06720	0.0542	0.121	

Signification codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1			
Permutation: free			
Number of permutations: 999			
<hr/>			
Goodness of fit:			
Subject ID	0.9365	0.001	***
Treatment timepoint	0.0127	0.936	
Treatment	0.0090	0.501	
Sex	0.0324	0.069	
Signification codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1			
Permutation: free			
Number of permutations: 999			

A) PCoA of UniFrac Distances**B) PCoA of wUniFrac Distances**

Supplementary Figure 1. Principal coordinates analysis using a) Unweighted and b) Weighted UniFrac distances.

Conclusions / Conclusiones



Conclusions

The following conclusions can be drawn for the reported results concerning the different bioactive fractions obtained from *L. edodes* using advanced extraction technologies. They have been organized as responses to each of the initially proposed objectives:

Objective 1. Production of extracts containing fungal sterols and ergocalciferol and evaluation of their hypocholesterolemic properties.

*Ergosterol-enriched extracts can be obtained using a pilot-scale supercritical CO₂ extraction plant (with yields up to 3.3% and ergosterol content up to 18% w/w). Ergosterol yields are highly influenced by both pressure and temperature utilized for extraction.

*UV-irradiation of SFE fractions lead to a successful conversion of ergosterol into ergocalciferol (vitamin D₂). The use of organic solvents and wide spectrum UV or UV-C lamps is encouraged. The irradiation of SFE extract is > 100 folds more effective than direct fruiting body irradiation.

*High amounts of ergosterol-enriched extracts can also be successfully produced using a large-scale supercritical CO₂ extraction plant (yields up to 0.6% and ergosterol content up to 53% w/w). Significant differences of ergosterol and derivatives levels are observed between the two separators where the extracts are recovered.

*Ergosterol and ergosterol-enriched extracts can be microemulsified and the process does not modify their properties as cholesterol displacers from dietary mixed micelles (DMM) in an *in vitro* digestion model.

*Ergosterol and ergosterol-enriched extracts with/without β -D-glucan-enriched (BGE) mixture can reduce cholesterol incorporation into DMM and, although no synergies are noticed, the BGE mixture can displace cholesterol from DMM.

*The ergosterol-enriched extracts exert no hypocholesterolemic activity in animal trials. However, *in vivo* experiments show that the BGE mixture can reduce serum cholesterol levels in hypercholesterolemic mice.

Objective 2. Production of extracts containing eritadenine and evaluation of its hypocholesterolemic properties.

*Eritadenine is present in significant amounts in mushrooms such as *Marasmius oreades* and *Amanita caesarea*. However, *L. edodes* almost doubles the amounts of the bioactive compound.

*Eritadenine is synthesized during the complete development of *L. edodes* fruiting bodies in all the mushroom tissues but particularly in their epidermis.

*The method of Afrin et al. (2016) (Chapter 2) to determine eritadenine is more accurate than the method of Enman et al. (2007) (Chapter 2).

*Grilling and gelling are recommended more than other culinary methods not only to maintain eritadenine levels but also because they enhance its bioaccessibility.

**In vivo* experiments in rats show that eritadenine consumption is safe, it reaches its target (liver) and it lowers the atherogenic index TC/HDL.

Objective 3. Production of β -D-glucan-enriched extracts and evaluation of their biological activities.

*Microwave-assisted extraction can be used to obtain polysaccharide-enriched fractions from *L. edodes* being 180 °C and 30 min extraction the optimal to extract 9.2% polysaccharides.

*Pre-treatment of shiitake with SFE followed by SWE (subcritical water extraction) or UAE (ultrasound assisted extraction) or combination of UAE + SWE are more effective methodologies than single or conventional extractions to obtain high polysaccharide yields. However, depending on the procedure, certain biological activities might be influenced.

*Large amount of β -D-glucan-enriched extracts can be successfully extracted using a pilot-scale extraction unit coupled to filtration devices. The nanofiltration membrane (Nanomax50) achieves an effective β -D-glucans enrichment after extraction and clarification. Apart from β -D-glucans, other bioactive molecules such as eritadenine can be concentrated.

*The fluorimetric method commonly used to quantify fungal (1→3)-β-D-glucans shows certain limitations. After certain adjustments in the protocol, it can be used only for detection and preliminary determination depending on the mushroom species and the sample complexity. Nevertheless, NMR analysis confirms that, indeed, the extracts obtained contain large concentrations of β-D-glucans with (1→3) and (1→3),(1→6) linkages plus a few other heteropolysaccharides. Their precise composition depends on the extraction technology and the selected parameters utilized.

*Two bioactive glucans can be purified, a (1→3)-α-glucan and a (1→3),(1→6)-β-D-glucan and a specific fraction can be isolated ((1→6)-β-D-glucan-rich fraction) with a few bioactivities but particularly with cytotoxic effects toward tumoral breast cells without interfering with normal breast cells growth.

*The different extracts obtained using the above mentioned technologies show high HMGCR inhibitory activity. They also show antioxidant and immunomodulatory properties according to *in vitro* tests.

Objective 4. Design of a scalable sequential procedure to optimize the extraction of several bioactive fractions from the same batch of shiitake fruiting bodies or by-products.

*A sequential extraction method can be successfully designed by chaining a first extraction from shiitake fruiting bodies or stipes (by-products) with cold water, the remaining material is submitted to a second extraction with SFE and the obtained residue is again submitted to a third extraction with hot water.

*Several bioactive compounds can be differentially extracted since extracts obtained with cold water contain high levels of water soluble β-D-glucans, chitooligosaccharides, eritadenine, lenthionine and hypotensive peptides. The SFE fraction contains fungal sterols and, after hot water extraction, two different β-D-glucan-enriched fractions can be obtained, one of them with large chitin concentrations. Mushrooms stipes contain the same bioactive compounds than the fruiting bodies.

*The highest antioxidant activity is recorded for the cold water extract followed by the hot water extract. The HMGCR inhibitors are preferentially extracted with cold water and the angiotensin converting enzyme (ACE) inhibitory activity is observed in cold and hot water extracts.

Objective 5. Demonstration of the biological activities of a designed functional food in a human trial.

*Using the pilot-scale extraction unit and other mentioned methodologies, the BGE mixture containing (besides β -D-glucans), eritadenine and ergosterol, can be prepared and integrated in the three utilized commercial food matrices to carry out the clinical trial.

*The BGE mixture does not lower cholesterol levels in subjects sera when compared to placebo nor show significant changes in pro-inflammatory cytokine concentrations. However, they reach the dietary fibre contents recommended by authorities as cardiovascular protective despite showing a less healthy general intake.

*The microbiome of the subjects consuming the BGE mixture is differently modulated than the placebo after the intervention time and some of the bacterial genera correlate with some cholesterol biomarkers. However, a clear conclusion concerning the real significance of these observations cannot be drawn since the complex interactions microbiome-host are still not completely understood.

Conclusiones

Los resultados expuestos en relación con las diferentes fracciones bioactivas obtenidas a partir de *L. edodes* utilizando tecnologías de extracción avanzadas han dado lugar a las conclusiones siguientes. Éstas han sido organizadas como respuesta a cada uno de los objetivos propuestos inicialmente:

Objetivo 1. Producción de extractos que contienen esteroides fúngicos y ergocalciferol y evaluación de sus propiedades hipocolesterolémicas:

*Utilizando una planta de extracción con CO₂ supercrítico a escala piloto, pueden obtenerse extractos enriquecidos en ergosterol (con rendimientos de hasta un 3,3% y contenido de ergosterol de hasta un 18% m/m). Los rendimientos de ergosterol obtenidos están fuertemente influenciados tanto por la presión como por la temperatura utilizada para la extracción.

*La irradiación UV de las fracciones SFE dan lugar a una conversión exitosa de ergosterol en ergocalciferol (vitamina D₂). Se recomienda el uso de disolventes orgánicos y lámparas UV de amplio espectro o UV-C. La irradiación de los extractos SFE es más de 100 veces más efectiva que la irradiación directa del cuerpo fructífero.

*Pueden producirse de forma exitosa grandes cantidades de extractos enriquecidos en ergosterol utilizando una planta de extracción con CO₂ supercrítico a mayor escala (con rendimientos de hasta 0.6% y contenido de ergosterol de hasta 53% m/m). Se observan diferencias significativas con respecto a los niveles de ergosterol y derivados entre los dos separadores en los que se recuperan los extractos.

*El ergosterol y los extractos enriquecidos en ergosterol pueden ser microemulsionados y el proceso no modifica su propiedad de desplazar el colesterol de las micelas mixtas de la dieta (DMM) en un modelo de digestión *in vitro*.

*El ergosterol y los extractos ricos en ergosterol con/sin la mezcla enriquecida en β -D-glucanos (BGE) pueden reducir la incorporación del

colesterol en las DMM y, aunque no se aprecian sinergias, la mezcla BGE es capaz de desplazar el colesterol de las DMM.

*Los extractos enriquecidos en ergosterol no presentan actividad hipocolesterolemica en ensayos animales. Sin embargo, los experimentos *in vivo* muestran que la mezcla BGE es capaz de reducir los niveles séricos de colesterol en ratones hipercolesterolemicos.

Objetivo 2. Producción de extractos que contienen eritadenina y evaluación de sus propiedades hipocolesterolemicas:

*La eritadenina está presente en cantidades significativas en setas tales como *Marasmius oreades* y *Amanita caesarea*. Sin embargo, *L. edodes* prácticamente duplica los niveles de este compuesto bioactivo.

*La eritadenina se sintetiza durante todo el desarrollo de los cuerpos fructíferos de *L. edodes* y en todos los tejidos de la seta, aunque particularmente en la epidermis.

*El método de Afrin et al. (2016) (Capítulo 2) para determinar eritadenina es más preciso que el método de Enman et al. (2007) (Capítulo 2).

*El asado y la gelificación son más adecuados que otros métodos culinarios no solo para mantener los niveles de eritadenina sino porque además mejorar su bioaccesibilidad.

*Los experimentos *in vivo* en ratas muestran que el consumo de eritadenina es seguro, que alcanza su diana (hígado) y que reduce el índice aterogénico colesterol total / colesterol HDL (TC/HDL).

Objetivo 3. Producción de extractos enriquecidos en β -D-glucanos y evaluación de sus actividades biológicas:

*La extracción asistida por microondas puede ser utilizada para obtener fracciones enriquecidas en polisacáridos a partir de *L. edodes*, siendo 180 °C y 30 min de extracción las condiciones óptimas para extraer un 9.2% de polisacáridos.

*El pretratamiento de shiitake con SFE seguido de SWE (extracción con agua subcrítica) o UAE (extracción asistida por ultrasonidos) o la combinación de

UAE + SWE son metodologías más efectivas que las extracciones individuales o convencionales a la hora de obtener altos rendimientos de polisacáridos. Sin embargo, dependiendo del procedimiento, ciertas actividades biológicas pueden verse afectadas.

*Grandes cantidades extractos enriquecidos en β -D-glucanos pueden extraerse exitosamente utilizando una unidad de extracción a escala piloto acoplada a equipos de filtración. La membrana de nanofiltración (Nanomax50) consigue un enriquecimiento efectivo de los β -D-glucanos tras la extracción y clarificación. Además de los β -D-glucanos, otras moléculas bioactivas como la eritadenina pueden ser concentradas.

*El método fluorimétrico comúnmente utilizado para cuantificar (1 \rightarrow 3)- β -D-glucanos fúngicos presenta ciertas limitaciones. Después de aplicar ciertos ajustes en el protocolo, se puede usar únicamente para la detección y determinación preliminar en función de la especie de seta y de la complejidad de la muestra. No obstante, los análisis de resonancia magnética nuclear (NMR) confirman que, de hecho, los extractos obtenidos contienen altas concentraciones de β -D-glucanos con enlaces (1 \rightarrow 3) y (1 \rightarrow 3),(1 \rightarrow 6), además de otros heteropolisacáridos. Su composición precisa depende de la tecnología de extracción y de los parámetros seleccionados.

*Es posible purificar dos glucanos bioactivos, un (1 \rightarrow 3)- α -glucano y un (1 \rightarrow 3),(1 \rightarrow 6)- β -D-glucano y aislar una fracción específica (fracción rica en (1 \rightarrow 6)- β -D-glucano) con varias bioactividades, particularmente con efectos citotóxicos sobre células tumorales de mama sin interferir con el crecimiento de células normales de mama.

*Los diferentes extractos obtenidos utilizando las tecnologías previamente mencionadas presentan actividad inhibidora de la HMGCR. También poseen propiedades antioxidantes e inmunomoduladoras de acuerdo con los test *in vitro*.

Objetivo 4. Diseño de un procedimiento escalable y secuencial para optimizar la extracción de varias fracciones bioactivas a partir del mismo lote de cuerpos fructíferos de shiitake o subproductos:

*Un método de extracción secuencial puede diseñarse exitosamente encadenando una primera extracción a partir de cuerpos fructíferos o pies (subproductos) de shiitake con agua fría, sometiendo el material remanente a una segunda extracción con SFE y sometiendo de nuevo el residuo obtenido a una tercera extracción con agua caliente.

*Varios compuestos bioactivos pueden ser extraídos de forma diferencial, ya que los extractos obtenidos con agua fría contienen altos niveles de β -D-glucanos solubles en agua, quitooligosacáridos, eritadenina, lentinina y péptidos bioactivos. La fracción SFE contiene esteroides fúngicos y, después de la extracción con agua caliente, se pueden obtener dos fracciones enriquecidas en β -D-glucanos, una de ellas con altas concentraciones de quitina. Los pies de las setas contienen los mismos compuestos bioactivos que los cuerpos fructíferos.

*La actividad antioxidante más alta se registra en el extracto obtenido con agua fría, seguido del extracto obtenido con agua caliente. Los inhibidores de la HMGCR son extraídos preferentemente con agua fría y la actividad inhibidora de la enzima convertidora de angiotensina (ACE) se observa en los extractos obtenidos con agua fría y caliente.

Objetivo 5. Demostración de la actividad biológica de un alimento funcional diseñado en un ensayo clínico en humanos:

*Mediante el uso de la unidad de extracción a escala piloto y otras tecnologías ya mencionadas, puede prepararse la mezcla BGE, que contiene (además de β -D-glucanos) eritadenina y ergosterol y se puede integrar en las tres matrices alimentarias comerciales utilizadas para llevar a cabo el ensayo clínico.

*La mezcla BGE no reduce los niveles séricos de colesterol en los sujetos en comparación con el placebo, ni muestra cambios significativos en las concentraciones de citoquinas pro-inflamatorias. Sin embargo, permite alcanzar las cantidades de fibra dietética recomendadas por las autoridades

como protección ante riesgo cardiovascular a pesar de presentar una ingesta general menos saludable.

*El microbioma de los sujetos que consumen la mezcla BGE se modula de forma diferente al del grupo placebo después del tiempo de intervención y algunos de los géneros de bacterias se correlacionan con determinados biomarcadores de colesterol. Sin embargo, no puede extraerse una conclusión clara en torno a la significancia real de estas observaciones, ya que las complejas interacciones microbioma-hospedador aún no pueden comprenderse en su totalidad.

About the author

Biography

Diego Morales was born in 1992 in Plasencia where he carried out the elementary and high school before moving to Salamanca to start his studies at the USAL (Universidad de Salamanca) in 2010. In 2014 he got his degree in Biotechnology and initiated a Master of Science at the UAM (Universidad Autónoma de Madrid), called ‘Agrochemistry and Novel Foods’. His first contact with scientific research was during this MSc since he worked in the Institute of Food Science Research (CIAL) with immunomodulating hydrolysates from egg white at the Alergenicity of Food Proteins and Peptides Group (Spanish Scientific Research Council, CSIC).

Afterwards, he obtained a contract (FPI) from the Ministry of Science, Innovation and Universities, starting his PhD in Food Science in 2015 and being part of the Department of Production and Characterization of Novel Foods (UAM, CIAL). Under the supervision of Dr. Cristina Soler-Rivas and Dr. Alejandro Ruiz-Rodríguez he focused his research on the utilization of advanced technologies to extract bioactive ingredients from *Lentinula edodes* and other edible mushrooms.

During his PhD and within the project called ‘REDUCOL2’ he was author and co-author of several research works that were published in scientific journals, books or communicated in national and international conferences. Moreover, he got the financial support to develop two scientific stays in the UFPR (Universidade Federal do Paraná) in Curitiba (Brasil, 2018) and the WUR (Wageningen University) in Wageningen (The Netherlands, 2019). These periods let him go deeper on the study of mushroom carbohydrates and the effect of the bioactive ingredients on human microbiome.

As member of the UAM, he was also involved in teaching activities and supervising experimental works developed by under-graduate and master students.

Besides, encouraged by his passion for science and scientific dissemination, he participated in many events and projects of technology transference to people with different backgrounds. Recently, he also created a scientific YouTube channel called ‘ScienceBeach_’ where he interviews young scientists.

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Hay que imaginarse a Sísifo feliz...

Albert Camus

Appendix



Vitamin D-enriched extracts obtained from shiitake mushrooms (*Lentinula edodes*) by supercritical fluid extraction and UV-irradiation

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UV-irradiation
Vitamin D₂

ABSTRACT

The combination of supercritical fluid extraction followed by UV-irradiation resulted in an interesting strategy to obtain ergosterol- and Vitamin D₂-enriched extracts from *Lentinula edodes* to design novel functional food. Extractions carried out following a specific experimental design pointed out that extraction yields and ergosterol concentrations were more influenced by pressure than by the extraction temperature although, high temperatures (55–75 °C) might induce transformation of ergosta-7,22-dienol and fungisterol into ergosterol. After extraction, the ergosterol-enriched extracts should be dissolved in methanol or ethanol and irradiated (25 °C, 4 cm) to partially transform ergosterol into vitamin D₂. Irradiation at 365 nm was less effective than at 254 nm. The fastest transformation was obtained using a UV lamp covering the complete UV spectrum for a maximum of 1 h. However, this lamp also induced vitamin D₄ formation although in lower amounts than vitamin D₂ or lumisterol₂ while with irradiation at 254 nm most of ergosterol was transformed into vitamin D₂.

Industrial relevance

This work describes a new method to enhance the vitamin D content of hypocholesterolemic extracts obtained from edible mushrooms such as *Lentinula edodes* (shiitake mushrooms). The generated extracts might be used to design novel functional foods by simple adjustment of the amount of ergosterol that wants to be transformed into vitamin D. Firstly, the mushrooms are submitted to supercritical fluid extraction (a process that can be up scaled since nowadays it is widely utilized for coffee decaffeination) to obtain ergosterol-enriched fractions. Then, they might be dissolved in GRAS solvents such as ethanol and irradiated with UV light to generate vitamin D. Irradiation of an extract obtained from the mushroom seems more effective than direct fruiting bodies irradiation, since > 100 folds vitamin D can be generated.

1. Introduction

Edible mushrooms contain many different hypocholesterolemic compounds such as β-glucans, fungal sterols, specific strain-dependent compounds, etc. that could be extracted and utilized to design bioactive

ingredients for novel functional foods. Several studies indicated that these molecules might modulate cholesterol homeostasis by inhibiting the endogenous cholesterol biosynthesis and impairing exogenous cholesterol absorption (Gil-Ramirez & Soler-Rivas, 2014; Guillamon et al., 2010). However, there might be many other indirect mechanisms involved in the regulation of serum cholesterol levels since, for instance, mushroom extracts containing eritadenine, an inhibitor of the S-adenosyl-L-homocysteine hydrolase (a key enzyme in the hepatic phospholipid metabolism) lowered total cholesterol levels *in vivo* (Sugiyama, Akachi, & Yamakawa, 1995). Vitamin D might also indirectly affect cholesterol levels (besides the other already known calcium- or parathyroid-related metabolic pathways) since low vitamin D status was associated with high total cholesterol levels and an increased risk of developing hyperlipidemia (Skaaby et al., 2012; Vitezova et al., 2015).

The different vitamin D structures are synthesized from provitamins D that are temporarily transformed by UV irradiation into previtamins D. The latter intermediate can generate tachysterols, lumisterols or vitamins D depending on light and temperature (Wittig, Krings, & Berger, 2013). The main vitamins D found in foods

Abbreviations: SFE, Supercritical fluid extraction; WS-UV, Wide-spectrum ultraviolet light; GC-FID-MS, Gas chromatography coupled to flame ionization detector and mass spectrometry; HPLC-DAD, High performance liquid chromatography coupled to diode array detection; HPLC-MS, High performance liquid chromatography coupled to mass spectrometry

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are ergocalciferol (or vitamin D₂), cholecalciferol (or vitamin D₃) and 22,23-dihydroergocalciferol (or vitamin D₄). The vitamin D form found in blood serum is generated in liver by cholecalciferol hydroxylation originating 25-hydroxycholecalciferol. However, some reports preferred to name it as 25-hydroxyvitamin D since ergocalciferol can also be bioavailable yielding 25-hydroxyergocalciferol and improving the levels of total 25-hydroxyvitamins D (Keegan, Lu, Bogusz, Williams, & Holick, 2013).

Mushrooms contain mainly vitamin D₂ (with traces of the other vitamins D (Keegan et al., 2013)) but their levels are largely dependent on environmental conditions. Those picked from the woods usually showed higher levels than indoor cultivated mushrooms. However, they all contain ergosterol (and other derivative sterols) because it is a constitutive compound in fungal hyphae as well as an ergocalciferol (vitamin D₂) precursor. Transformation of ergosterol into vitamin D₂ takes place during the development of fruiting bodies exposed to light however, it can be reproduced *in vitro* by exposing their caps or gills to UV-light (Jasinghe & Perera, 2005; Mau, Chen, & Yang, 1998; Slawinska et al., 2016). *Pleurotus* sp. fruiting bodies increased their vitamin D₂ content from almost 0 to approx. 60 or 200 µg/g depending on the analyzed strain after 2 h UV-B irradiation (Huang, Lin, & Tsai, 2015). Lower irradiation times yielded lower transformation but still 30 min exposure increased vitamin D₂ levels in *A. bisporus* and *L. edodes* up to respectively 119.21 and 59.89 µg/g (Slawinska et al., 2016). Exposure of gills facing UV-A source induced 4.4 fold more transformation of ergosterol into vitamin D₂ than cap exposure (Jasinghe & Perera, 2005) being UV-B irradiation more effective than UV-A or UV-C.

Recent works indicated that ergosterol enriched fractions obtained from mushrooms using supercritical fluid extractions (SFE) were able of lowering cholesterol levels in hypercholesterolemic mice (Caz et al., 2016) however, they might be even more effective if ergosterol is partly transformed into vitamin D₂ as noticed on hypercholesterolemic patients treated with statins after vitamin D supplementation (Qin, Zhao, Chen, Yin, & Wang, 2015). Therefore, in this work a new method to extract ergosterol using SFE and convert it into vitamin D₂ is described. The UV irradiation was carried out once the extract was generated and not on the mushroom fruiting body, as has traditionally been done.

2. Material and methods

2.1. Biological material and reagents

Powdered *Lentinula edodes* S. (Berkeley) fruiting bodies were purchased from Glucanfeed S.L. (La Rioja, Spain) and stored in darkness at −20 °C until further use. Obtained powder showed a particle size lower than 0.5 mm and a moisture content lower than 5%.

Solvents as hexane (95%), chloroform (HPLC grade) and methanol (HPLC grade) were obtained from LAB-SCAN (Gliwice, Poland) and absolute ethanol and sea sand from Panreac (Barcelona, Spain). Potassium hydroxide, ascorbic acid and BHT (2,6-Di-tert-butyl-*p*-cresol) as well as hexadecane, ergosterol (95%), ergocalciferol (99%) (vitamin D₂) and cholecalciferol (98%) (vitamin D₃) were purchased from Sigma-Aldrich Química (Madrid, Spain). The CO₂ (99.99% purity) was supplied by Air-Liquid España, S.A. (Madrid, Spain). All other reagents and solvents were used of analytical grade.

2.2. Supercritical fluid extractions (SFE)

Supercritical fluid extractions with CO₂ were carried out in a plant (Thar Technology, Pittsburgh, PA, USA, model SF2000) comprising a 2 L cylinder extraction cell and two different separators (S1 and S2) of 0.5 L capacity each with independent control of temperature and pressure. The extraction vessel had a ratio of 5.5 height/diameter. A detailed explanation of the experimental device can be found elsewhere (García-Risco, Vicente, Reglero, & Fornari, 2011). The extraction cell

Table 1

Central composite design (2² + star design) of supercritical extractions from *Lentinula edodes*.

Run	Variables	
	Temperature (°C)	Pressure (bar)
1	33.8	225.0
2	70.0	100.0
3	55.0	225.0
4	55.0	225.0
5	76.2	225.0
6	40.0	100.0
7	40.0	350.0
8	55.0	225.0
9	55.0	401.8
10	70.0	350.0
11	55.0	48.2

was filled with shiitake powder (253 g) and washed sea sand (1100 g) in a ratio of 1:1 (v/v). The temperature of separators 1 and 2 was set to 40 °C for all the experimental assays and the pressure of S1 and S2 was maintained at 60 bar for all the extractions. The CO₂ flow was set at 3.6 kg/h and during the total extraction time (3 h) it was recirculated. The compounds extracted in both separators were washed with ethanol and immediately submitted to concentration until dryness on a rotary vacuum evaporator. The dried extracts were stored at −20 °C until further analysis. Extracted dry matter content was measured to calculate the extraction yields. Extraction yields were expressed as percentage of dry matter (in grams) obtained from 100 g of dry raw material utilized for extraction.

In order to optimize the extraction method to obtain sterol-enriched fractions, parameters such as extraction pressure and extraction temperature were tested following a central composite design (2² + star design). All the experiments (see Table 1) were fully randomized to provide protection against the effect of lurking variables. Values for extraction temperature and pressure ranged respectively from 33.8 to 76.2 °C and 48.2 to 401.8 bar, with star points corresponding to 33.8 and 76.2 °C in the case of temperature, and 48.2 and 401.8 bar in the case of pressure.

2.3. UV-irradiation of SFE extracts

Sterol-enriched extracts obtained by SFE and lyophilized shiitake mushrooms were submitted to UV-irradiation using two different lamps: a Höhensonne 100 quartz lamp from Original Hanau (Hanau, Germany) that emits UV radiation covering a wide light spectrum (200–700 nm) since it coupled an IR rod (WS-UV); and a VL-4.LC lamp from Vilber Lourmat (Eberhardzell, Germany) that can irradiate specifically at 254 (UV-C) or 365 nm (UV-A).

Powdered *L. edodes* fruiting bodies (50 mg) were mixed with 3 mL of different solvents (water, methanol and ethanol) in 2.5 cm diam. × 8 cm height cylindrical vessels, and exposed uncovered to the radiation under vigorously shaking at a distance of 26 (Höhensonne lamp) or 14 (VL-4 lamp) cm for different incubation times (0, 15, 30, 60 and 120 min). Other distances to the lamp were also tested and therefore, the vials were placed at 4, 14 and 24 cm far from the UV source. Similarly, the fractions obtained after SFE extractions (12 mg) were dissolved and treated as previously mentioned for the powdered fruiting bodies.

2.4. GC-FID-MS analysis

Fungal sterols from both shiitake mushrooms and SFE fractions (irradiated and non-irradiated) were extracted following the procedure described by Gil-Ramirez et al. (2013). The unsaponified fractions obtained (6 mg/mL) were injected into an Agilent 19091S-433 capillary

column (30 m × 0.25 mm ID and 0.25 µm phase thickness). The column was connected to a 7890A System gas chromatograph (Agilent Technologies, USA) including a G4513A auto injector and a 5975C triple-Axis mass spectrometer detector. The injector and detector conditions as well as the column temperature program were those described by Gil-Ramirez et al. (2013). Ergosterol was used as standard to validate the GC method, using hexadecane (10% v/v) as internal standard.

GC-MS database identified the obtained peaks in concordance with previous studies (Gil-Ramirez et al., 2013; Jasinghe & Perera, 2005; Teichmann, Dutta, Staffas, & Jägerstad, 2007). The major detected sterols were ergosterol (ergosta-5,7,22-trien-3β-ol) (RT = 12.6 min), ergosta 7,22-dienol (RT = 12.8 min), ergosta-5,7-dienol (RT = 13.1 min) and ergosta-7-enol (fungisterol) (RT = 13.3 min).

2.5. HPLC-DAD and HPLC-MS analyses

The unsaponified fractions obtained as previously described from both shiitake mushrooms or irradiated and non-irradiated SFE fractions were injected (20 µL) into a Varian HPLC, model 920-LC Galaxy, with a diode array (PAD) detector. Reverse phase chromatographic separation was performed with a Carotenoid C30 analytical column (250 × 4.6 mm, 5 µm) from YMC Europe (Dinslaken, Germany). Solvents utilized as mobile phase were 85% methanol (v/v) (A) and ethanol (B). They were mixed following the gradient: 5% B during 2 min, up to 40% B in 5 min and then maintained 15 min, up to 90% B in 5 min and maintained 5 min more. The flow rate was 1 mL/min and the oven temperature 50 °C. The absorbance changes were followed by a UV-VIS DAD and 265 nm was selected for quantification. Vitamin D₂ (R.T. = 16.2 min) and vitamin D₃ (R.T. = 16.5 min) were injected as standards obtaining chromatograms similar to Wittig et al. (2013). Nevertheless, to identify vitamin D-related structures, samples were also injected (using the above described method and column) into an Agilent 1100 series liquid chromatograph equipped with a PDA detector and directly coupled to an ion trap mass spectrometer (Agilent ion trap 6320) via an atmospheric pressure chemical ionization (APCI) interface. The selected parameters and conditions were: positive ionization mode, capillary voltage, −3.5 kV; drying temperature, 350 °C; vaporizer temperature, 400 °C; drying gas flow rate, 5 L/min; corona current, 4000 nA; nebulizer gas pressure, 60 psi. Full scan was acquired in the range from *m/z* 50 to 2200.

Samples were injected in duplicate and ergocalciferol (vitamin D₂) was used as standard for the quantitative determination of vitamins D and derivative compounds.

2.6. Statistical data analyses

The one-way ANOVA as well as the Durbin-Watson statistic tests was used to determine the statistical significance of the extraction pressure and temperature on the percentage of ergosterol extracted. Significance was set at *P* < 0.05. Calculations were made using StatGraphics Centurion XVII.I (Statpoint Technologies, Inc., Virginia, USA) software.

The rest of experimental data was analyzed for statistical significance by one-way ANOVA followed by Tukey's multiple comparison test (*α* = 0.05) using Prism GraphPad 5.03 software (GraphPad Software Inc., San Diego, CA, USA).

3. Results and discussion

3.1. SFE extraction of sterols-enriched fractions

Fruiting bodies from *Lentinula edodes* were submitted to supercritical CO₂ extractions without co-solvent since previous results carried out with both *L. edodes* and *A. bisporus* stated that mixtures including 5, 10 or 15% ethanol (v/v) yielded extracts with higher dry

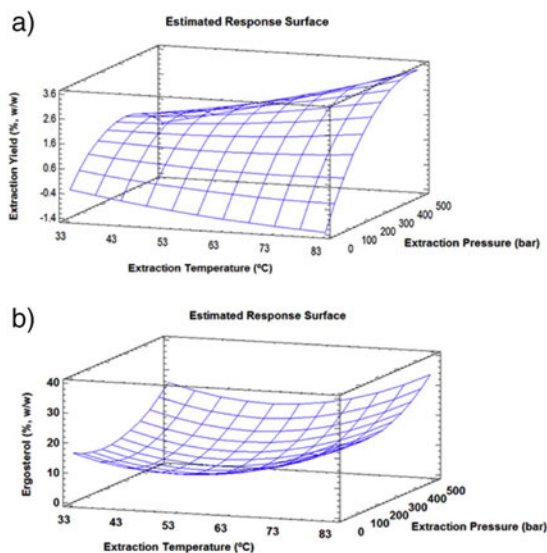


Fig. 1. Response Surface Plot for a) extraction yield and b) ergosterol concentration obtained after SFE extractions (expressed in % w/w).

matter but lower sterol contents (Gil-Ramirez et al., 2013; Kitzberger, Smania, Pedrosa, & Ferreira, 2007 and 2009). Moreover, the use of other solvents such as dichloromethane or ethyl acetate did not improve the extracted amounts (Kitzberger et al., 2007). Thus, two extraction variables such as pressure and temperature were tested following the previously described experimental design (Table 1) to estimate the optimal combinations.

To study the level of significance of each factor an analysis of variance (ANOVA) was performed for two selected responses (extraction yield and percentage of ergosterol extracted). To be able of describing the effects of the different factors and interactions in the response, only the significant factors were chosen (95% confidence level).

The ANOVA analysis of the extraction yield showed that only the factor extraction pressure had a *P*-value < 0.05, indicating that it was significantly different from zero at the 95% confidence level. The Response Surface Plot for the extraction yield (% w/w) (Fig. 1a) indicated that the extraction yields were much more influenced by the extraction pressure, being the highest of 3.28%, at the conditions of 350 bar and 70 °C. These results were in concordance with those Kitzberger et al. (2009), which reported that high pressures combined with the increase of the operational temperature led to the enhancement of the extraction yield of shiitake oil.

However, results obtained with conditions such as 225 bar and 75 °C allowed recovery yields of approx. 2.3% (w/w) being higher than those obtained in previous reports (approx. 1%) where lower temperatures and/or similar or higher pressures were selected (Kitzberger et al., 2009). Mazzutti, Ferreira, Riehl, Smania, and Martinez (2012) reported yields of 1.19% when working with *Agaricus brasiliensis* at 300 bar and 50 °C. Previous results using *Agaricus bisporus* as raw material achieved yields of approx. 0.6% at extraction temperatures of 40 °C regardless of the pressure selected (90, 180 and 300 bar) (Gil-Ramirez et al., 2013).

In the case of ergosterol percentage (the grams of ergosterol extracted from 100 g of mushroom powder), both the extraction pressure and the extraction temperature had *P*-values < 0.05, indicating that they were significantly different from zero at the 95% confidence level. The regression equation fitting to the data was:

$$[\text{Ergosterol}] = 48.4066 - 1.42215 \cdot \text{Temperature} + 0.03441$$

$$* \text{Pressure} + 0.01362 \cdot \text{Temperature}^2 - 0.00001 \cdot \text{Temperature}$$

$$* \text{Pressure} + 0.00011 \cdot \text{Pressure}^2$$

The Response Surface Plot obtained by a graphical representation of the fitted equation (Fig. 1b) showed the behavior of the response as a function of the different factors values. An increase in both the extraction temperature and pressure led to an increase in the percentage of ergosterol in the extracts. The experimental condition of 350 bar and 70 °C provided the highest ergosterol percentage (18% w/w) corresponding to 180 mg/g dw (dry weight). This may be explained by the combined effect of a high CO₂ density (0.83 g/cm³) plus the vapor pressure at these conditions (Ghoreishi, Kamali, Ghaziaskar, & Dadkhah, 2012; Kamali, Aminimoghadamfarouj, Golmakani, & Nematollahi, 2015). The ergosterol content of the obtained fractions ranged from 87 mg/g dw (at 85 bar 55 °C) up to 170.6 mg/g at 350 bar 70 °C.

Fraction containing 18% ergosterol (w/w) included other ergosterol derivatives such as fungisterol (2%), ergosta 7,22-dienol (1.7%) and ergosta 5,7-dienol (0.2%). Increase of pressure did not largely influence the extraction of the other fungal sterols however, an increase of temperature might induce the transformation of ergosta-7,22-dienol and fungisterol into ergosterol. In extractions obtained at 225 bar, an increase in temperature from 35, 55 to 75 °C led to a rise in the ergosterol levels of respectively 76, 79 and 82% of the total extracted sterols, and to a decrease in ergosta-7,22-dienol levels of respectively 11, 10 and 8% and of 12, 10 and 9% in the case of fungisterol. On contrary, levels of ergosta-5,7-dienol remained constant but they only represented the 1% of the total extracted sterols.

3.2. Effect of the UV-lamp utilized for SFE-extracts irradiation

The powdered *L. edodes* strain utilized in this study contained ergosterol but no ergocalciferol (vitamin D₂) (Fig. 2). When the mushroom powder was directly exposed to UV-C irradiation (254 nm), ergosterol levels decreased 16% while vitamin D₂ increased. However, when the powder was suspended in methanol, stirred and irradiated, ergosterol levels were reduced 27% and vitamin D₂ levels increased 6.6 folds compared with initial values. These results indicated that irradiation of mushroom powder within a medium was more effective than dry irradiation and even more than direct fruiting body irradiation (if compared with levels previously reported). Fresh *L. edodes* fruiting bodies irradiation for 1 h but at 310 nm (UV-B) induced the formation of 0.004 mg/g vitamin D₂ (Mau et al., 1998) and 2 h incubation yielded 0.015 mg/g (Huang et al., 2015). If they were placed

with their gills facing the UV source for 30 min at 20 °C, 0.029 mg/g vitamin D₂ were generated while irradiation of dried fruiting bodies induced up to 0.06 mg/g (Slawinska et al., 2016).

Thus, the irradiation of the ergosterol-enriched extracts was carried out using methanol as solvent for two reasons: firstly, because after the above described comparison, the use of an organic solvent was more effective than using none or water to induce vitamin D₂ biosynthesis. The higher solubility of sterol and vitamin D derivatives in organic mixtures might positively influence transformation yields. Secondly, because the oily fractions obtained after SFE extraction were easier solubilized in organic solvents rather than in aqueous mixtures.

The SFE extract selected for irradiation studies was the one obtained after SFE extraction at 350 bar and 70 °C due to its higher ergosterol concentration compared to the ones obtained under other experimental conditions. This extract initially contained 216.9 mg/g fungal sterols where ergosterol represented 82.9% (w/w) of total sterols, ergosta-7,22-dienol and fungisterol were present in lower concentrations (respect. 7.7 and 7.6%), and ergosta-5,7-dienol was also detected but in very low quantities (1.8%) (Table 2). When the extract was solubilized and irradiated with the lamp able to supply a wide spectrum of UV light (WS-UV), a large reduction in the ergosterol level was noticed until its complete degradation after 2 h incubation (Fig. 3). This compound was rapidly transformed into vitamin D₂ since its level was increasing with the irradiation time until 1 h. Afterwards, the UV-light exposure seemed detrimental also for the vitamin perhaps, the heat generated by the IR rod coupled to the UV-lamp was excessive. IR irradiation was complementary used because heat enhanced UV effect enhancing the transformation of previtamins D into vitamins D rather than into tachysterols or lumisterols (Wittig et al., 2013). Levels of the other derivative sterols were also influenced and drastically reduced after 1 h irradiation. They were not expected to be transformed into any other compound due to the UV-light therefore, their reduction might also suggest a negative influence of the heat generated by the IR rod.

Irradiation with UV-A (365 nm) induced almost no transformation of ergosterol into vitamin D₂. However, the procedure still yielded more vitamin than described in other reports where the irradiation was carried out directly on mushroom fruiting bodies. SFE extract irradiation for 1 h yielded 0.24 mg/g vitamin D₂ and after 2 h the amount increased up to 1.13 mg/g while the largest amount obtained after 2 h of fruiting bodies UV-A irradiation (with gills facing the source) was 0.023 mg/g (Jasinghe & Perera, 2005). Nevertheless, UV-C irradiation (254 nm) of SFE extracts was more effective than UV-A because after 2 h UV-C irradiation almost half of the ergosterol content was transformed into vitamin D₂ that doubled its levels. Levels of the other ergosterol derivatives remained constant within the irradiation period independently of the wavelength utilized (Table 2).

Apparently, the UV-lamps utilized also stimulated production of different vitamins D and photoisomers (Table 3). According to HPLC-MS analysis, generated compounds were in concordance with those previously reported by Wittig et al. (2013). Previtamin D₂ formation could be noticed after 15 and 60 min when irradiated with respect. UV-C and the WS-UV lamps. But probably, it was further transformed since its levels were reduced further in time concomitantly with an increase of its derivatives such as vitamin D₂, lumisterol₂ and tachysterol₂. Lumisterol₂ was after vitamin D₂ the second previtamin D₂-derivative generated. Its levels were only approx. 3 fold lower than vitamin D₂ after 120 min irradiation in extracts irradiated with UV-C or after 60 min when irradiation was carried out with WS-UV lamps. The transformation of previtamin D₂ into tachysterol₂ was not stimulated since only low concentrations could be noticed after 60 min WS-UV irradiation. However, vitamin D₄ levels increased up to 60 min when the irradiation was carried out with the WS-UV lamp and was almost not detected with UV-C irradiation. Previtamin D₄, tachysterol₄ and lumisterol₄ were also increased although at lower concentrations than vitamin D₄ during 60 min with WS-UV irradiation.

Since irradiation with WS-UV lamp was promoting generation of

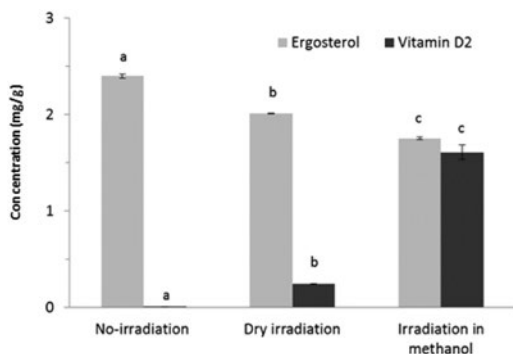


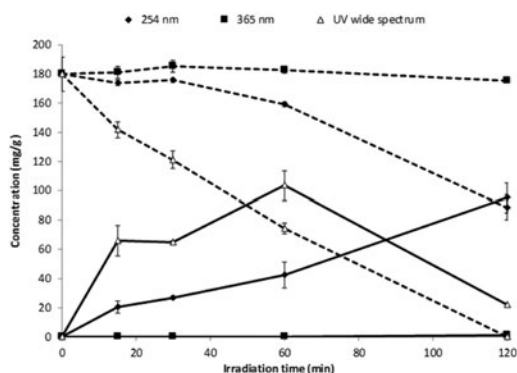
Fig. 2. Levels of ergosterol and vitamin D₂ (mg ergosterol or vitamin D₂/g mushroom powder) in powdered Shiitake fruiting bodies non-irradiated and irradiated with UV-C for 1 h at 50 °C. Irradiation was carried out directly on mushroom powder (dry irradiation) or suspended in methanol. ^{a-c} Different letters denote significant differences ($P < 0.05$) between values of the same series.

Table 2

Fungal sterols content in the SFE extract irradiated for 2 h using different UV-lamps.

Lamp	Time (min)	Ergosterol (mg/g)	Ergosta7,22-dienol (mg/g)	Fungisterol (mg/g)	Ergosta5,7-dienol (mg/g)	Total sterols (mg/g)
UV-C (254 nm)	0	179.89 ± 11.72 ^a	16.70 ± 0.82 ^a	16.50 ± 0.79 ^b	3.80 ± 0.23 ^a	216.89
	15	174.04 ± 2.00 ^a	8.93 ± 0.55 ^b	17.46 ± 0.23 ^b	3.34 ± 0.60 ^a	203.77
	30	175.82 ± 1.33 ^a	13.71 ± 0.21 ^{ab}	23.90 ± 2.44 ^a	7.13 ± 2.42 ^a	220.57
	60	159.26 ± 1.50 ^a	11.03 ± 0.03 ^b	18.41 ± 1.72 ^{ab}	3.74 ± 0.69 ^a	192.44
	120	88.75 ± 8.82 ^b	10.29 ± 0.53 ^b	22.78 ± 3.45 ^a	3.90 ± 1.04 ^a	125.72
UV-A (365 nm)	0	179.89 ± 11.72 ^a	16.70 ± 0.82 ^a	16.50 ± 0.79 ^a	3.80 ± 0.23 ^a	216.89
	15	181.34 ± 4.18 ^a	11.77 ± 3.13 ^a	24.51 ± 4.65 ^a	4.55 ± 0.77 ^a	222.17
	30	185.51 ± 4.19 ^a	13.26 ± 2.38 ^a	24.08 ± 3.53 ^a	4.68 ± 1.07 ^a	227.53
	60	182.87 ± 2.19 ^a	13.22 ± 2.19 ^a	22.86 ± 3.30 ^a	3.63 ± 0.61 ^a	222.58
	120	175.41 ± 0.24 ^a	11.03 ± 0.86 ^a	21.62 ± 0.94 ^a	3.79 ± 0.54 ^a	211.86
WS-UV	0	179.89 ± 11.72 ^a	16.70 ± 0.82 ^a	16.50 ± 0.79 ^b	3.80 ± 0.23 ^a	216.89
	15	141.74 ± 5.45 ^b	9.85 ± 0.13 ^b	17.95 ± 0.70 ^b	3.31 ± 0.68 ^a	172.85
	30	121.02 ± 6.00 ^b	16.55 ± 2.25 ^a	24.44 ± 1.27 ^a	1.79 ± 0.36 ^a	163.80
	60	74.40 ± 3.60 ^c	9.74 ± 1.71 ^b	17.42 ± 0.22 ^b	1.26 ± 0.04 ^a	102.83
	120	n.d. ^d	n.d. ^c	2.34 ± 0.30 ^c	n.d. ^b	2.34

n.d. = not detected.

^{a-d} Different letters denote significant differences ($P < 0.05$) between different times of exposure for the same compound and the same lamp.**Fig. 3.** Effect of UV light using different lamps on ergosterol (dashed line) and vitamin D₂ (solid line) levels during 2 h irradiation. Concentration is expressed in mg ergosterol or vitamin D₂/g SFE extract.

other structures (vitamin D₄ -related) and the aim of the work was to increase the level of vitamin D₂ but avoiding the complete ergosterol transformation (because of its hypocholesterolemic properties) the WS-UV lamp was discarded and the UV-C lamp was selected to carry out

further experiments.

3.3. Effect of the solvent and temperature utilized for SFE-extracts irradiation

Transformation of ergosterol from SFE extract into vitamin D₂ under UV-C light was also studied using different solvents. After 1 h irradiation (at 50 °C), vitamin D₂ was slightly synthesized if water was used as reaction medium (Fig. 4). Addition of methanol up to different percentages improved the transformation ratio but up to levels significantly lower than when irradiation was carried out using 100% organic solvents such as methanol or ethanol suggesting that solubilization of the SFE extract was essential for the proper vitamin D₂ generation.

Because of the detrimental effect noticed on sterol content and vitamin D₂ transformation after prolonged WS-UV irradiation, the effect of temperature was also studied on SFE extracts irradiated with the UV-C lamp to ensure that the observed degradation could be due to the heat generated by the IR rod and to estimate the optimal temperature needed to enhance the vitamin D₂ generation. However, no significant differences were found after 1 h UV-C irradiation if temperatures were maintained from 25 up to 50 °C (Fig. 5). Other tested temperatures (1 h) such as 30, 40 and 60 °C did not significantly improve speed of vitamin D generation (as also noticed by Wittig et al. (2013) after irradiation of

Table 3Vitamins D and photoisomers detected in the SFE extract during 2 h irradiation using different UV-lamps. Previtamin D₂ (PRE₂), tachysterol₂ (T₂), lumisterol₂ (L₂), vitamin D₂ (V₂), provitamin D₄ (PRO₄), previtamin D₄ (PRE₄), tachysterol₄ (T₄), lumisterol₄ (L₄) and vitamin D₄ (V₄).

Lamp	Time (min)	PRE ₂ (mg/g)	T ₂ (mg/g)	L ₂ (mg/g)	V ₂ (mg/g)	PRO ₄ (mg/g)	PRE ₄ (mg/g)	T ₄ (mg/g)	L ₄ (mg/g)	V ₄ (mg/g)
UV-C (254 nm)	0	n.d. ^a	n.d. ^a	n.d. ^a	n.d. ^a	n.d. ^b	n.d. ^a	n.d. ^a	n.d. ^b	n.d. ^a
	15	n.d. ^a	n.d. ^a	2.53 ± 0.86 ^a	20.54 ± 4.02 ^b	n.d. ^b	n.d. ^a	n.d. ^a	0.38 ± 0.42 ^b	n.d. ^a
	30	n.d. ^a	n.d. ^a	9.34 ± 0.00 ^a	26.81 ± 0.97 ^b	1.13 ± 0.00 ^a	n.d. ^a	n.d. ^a	1.67 ± 0.00 ^a	n.d. ^a
	60	0.10 ± 0.15 ^a	n.d. ^a	12.38 ± 3.72 ^a	42.38 ± 8.84 ^b	n.d. ^b	n.d. ^a	0.27 ± 0.22 ^a	0.16 ± 0.22 ^b	n.d. ^a
	120	n.d. ^a	n.d. ^a	32.74 ± 24.58 ^a	95.10 ± 10.23 ^a	n.d. ^b	0.58 ± 0.77 ^a	2.03 ± 2.07 ^a	0.70 ± 0.36 ^a	0.13 ± 0.19 ^a
UV-A (365 nm)	0	n.d. ^a	n.d. ^a	n.d. ^a	n.d. ^a	n.d. ^a	n.d. ^a	n.d. ^a	n.d. ^a	n.d. ^a
	15	n.d. ^a	n.d. ^a	n.d. ^a	n.d. ^a	n.d. ^a	n.d. ^a	n.d. ^a	0.26 ± 0.37 ^a	n.d. ^a
	30	n.d. ^a	n.d. ^a	n.d. ^a	n.d. ^a	n.d. ^a	n.d. ^a	n.d. ^a	0.55 ± 0.40 ^a	n.d. ^a
	60	0.41 ± 0.58 ^a	n.d. ^a	n.d. ^a	0.24 ± 0.34 ^a	n.d. ^a	n.d. ^a	n.d. ^a	0.22 ± 0.31 ^a	n.d. ^a
	120	0.33 ± 0.46 ^a	n.d. ^a	0.68 ± 0.96 ^a	1.13 ± 0.31 ^a	n.d. ^a	n.d. ^a	n.d. ^a	0.68 ± 0.37 ^a	n.d. ^a
WS-UV	0	n.d. ^c	n.d. ^a	n.d. ^c	n.d. ^c	n.d. ^a	n.d. ^a	n.d. ^b	n.d. ^b	n.d. ^c
	15	2.18 ± 0.00 ^a	n.d. ^a	27.78 ± 0.09 ^b	65.95 ± 10.33 ^b	0.03 ± 0.04 ^a	0.63 ± 0.04 ^a	1.01 ± 0.57 ^b	1.32 ± 0.55 ^a	1.05 ± 0.32 ^c
	30	1.43 ± 0.00 ^b	0.11 ± 0.16 ^a	35.89 ± 4.63 ^a	64.75 ± 1.34 ^b	n.d. ^a	0.83 ± 1.18 ^a	1.34 ± 0.08 ^a	1.36 ± 0.02 ^a	3.43 ± 0.16 ^b
	60	n.d. ^c	2.09 ± 1.16 ^a	45.24 ± 3.97 ^a	103.45 ± 10.02 ^a	n.d. ^a	1.65 ± 0.04 ^a	2.18 ± 0.04 ^a	1.53 ± 0.08 ^a	7.97 ± 0.44 ^a
	120	n.d. ^c	0.82 ± 0.31 ^a	2.45 ± 0.11 ^c	22.10 ± 0.97 ^c	n.d. ^a	n.d. ^a	n.d. ^b	0.20 ± 0.12 ^b	0.38 ± 0.19 ^c

n.d. = not detected.

^{a-c} Different letters denote significant differences ($P < 0.05$) between different times of exposure for the same compound and the same lamp.

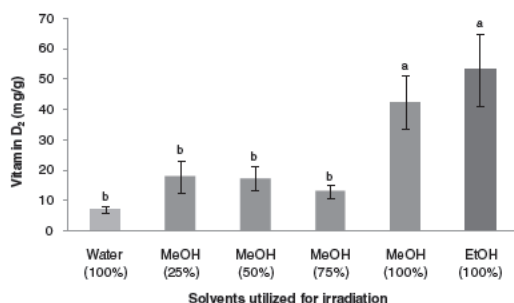


Fig. 4. Influence of the different solvents (utilized to dissolve SFE extract for UV irradiation) in vitamin D₂ levels. ^{a,b} Different letters denote significant differences ($P < 0.05$).

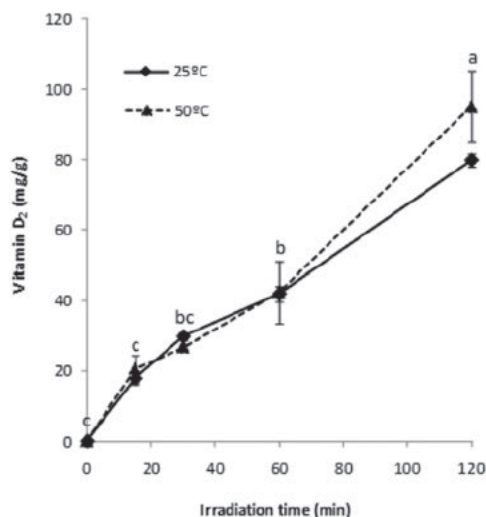


Fig. 5. Influence of the different temperatures (utilized during SFE extract UV irradiation) in vitamin D₂ levels. ^{a,c} Different letters denote significant differences ($P < 0.05$) between different irradiation times at the same temperature (25 °C or 50 °C).

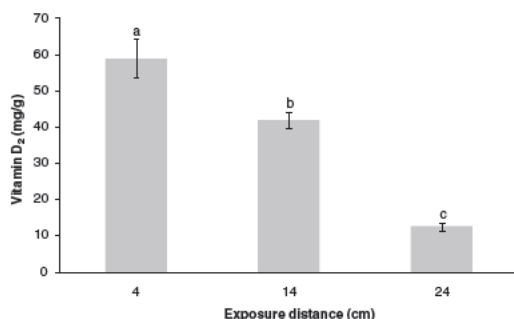


Fig. 6. Influence of the different distances from the UV lamp (utilized during SFE extract UV irradiation) in vitamin D₂ levels. ^{a,c} Different letters denote significant differences ($P < 0.05$).

Oyster mushrooms fruiting bodies). However, after 2 h irradiation of the SFE extract at 50 °C, a slightly higher amount of vitamin D₂ was obtained than at 25 °C.

3.4. Effect of the distance to UV source

The optimal distance to the UV source was also investigated because previous studies placed mushroom fruiting bodies at many different distances (from 10 up to 30 cm) with no further testing to study whether they were the more adequate position for irradiation. For instance, Huang et al. (2015) irradiated Oyster mushrooms with a UV-B lamp at 25 °C, for 2 h, at 19 cm far from the light and obtained 69 µg/g vitamin D while Wittig et al. (2013) irradiated the same mushrooms with a similar UV-B lamp at 20 and 30 °C at 10 cm from the light and obtained larger amounts (80 µg/g) with only 10 min irradiation. Differences between both experiments could be due to the fact that the latter mushrooms were placed closer to the UV lamp. Thus, SFE extracts were dissolved in methanol and placed at three different positions from the lamp (Fig. 6). Results indicated that the transformation of ergosterol into vitamin D₂ was enhanced when the vials were placed closer to the UV source since at 4 cm distance almost 5 fold more vitamin D₂ was obtained than at 24 cm after 1 h exposure.

4. Conclusions

Fractions containing up to 18% (w/w) ergosterol and other ergosterol derivatives can be obtained by supercritical fluid extractions from *Lentinula edodes*. They can be further processed to induce partial transformation of this provitamin D₂ into vitamin D₂ by UV-light irradiation. Then, the SFE extracts should be dissolved in organic solvents such as methanol or ethanol, exposed at room temperature under WS-UV or UV-C rather than UV-A light and as closer as possible to the UV source. WS-UV irradiation also induced vitamin D₄ formation although in lower amounts than vitamin D₂ or lumisterol₂. However, if WS-UV lamp is couple to IR rod, exposures longer than 1 h are not encouraged due to detrimental effect on ergosterol and vitamin D₂ contents. This procedure to generate vitamin D₂ enriched extracts is > 100 folds more effective than direct fruiting body irradiation followed by extraction.

Conflict of interest

None.

Acknowledgments

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In vitro and *in vivo* testing of the hypocholesterolemic activity of ergosterol- and β -glucan-enriched extracts obtained from shiitake mushrooms (*Lentinula edodes*)

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Herein, a supercritical extraction plant (with a 6 L extraction cell) was successfully used to obtain ergosterol-enriched extracts from *Lentinula edodes* under the following conditions: a temperature of 40 °C, pressure of 225 bar, reaction time of 1–5 h, and the flow rate of 20 L h⁻¹ for recirculated CO₂. Moreover, ergosterol (ERG) and the SFE extract (SFE) with highest ergosterol concentration were microemulsified and submitted to *in vitro* digestion to study their ability to displace cholesterol from dietary mixed micelles (DMMs). ERG was also mixed with a β -glucan-enriched (33.5%) extract (BGE) obtained from *L. edodes* to investigate the synergies between them; the results indicated that all these extracts (including BGE without ERG) could reduce the cholesterol levels in the DMMs. However, when ERG and SFE were simultaneously administered to mice with a hypercholesterolemic diet, no significant differences in the serum cholesterol levels were detected as compared to the case of the control. However, when only BGE was administered to another mice model previously induced with hypercholesterolemia, significant reduction in the cholesterol levels was noticed.

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1. Introduction

High levels of total cholesterol (TC) and particularly LDL-cholesterol constitute one of the most relevant risk factors of cardiovascular diseases that are still leading the causes of death, particularly in developed countries. However, before treatment with therapeutic drugs, for patients with milder hypercholesterolemia, regular consumption of specific functional foods may be more desirable; nowadays, products, including phytosterols or cereal β -glucans, exhibit health benefits as hypocholesterolemic products approved by most of the regulatory institutions such as the European Food Safety Authority (EFSA) and Food and Drugs Administration (FDA), and they are easily found in the supermarkets; however, novel or/and more effective products still need to be developed.^{1,2}

Edible mushrooms have been reported as one of the foods with potential hypocholesterolemic activities.³ They contain fungal sterols that (as noticed for phytosterols) may displace cholesterol from dietary mixed micelles (DMMs).⁴ Dietary cholesterol is mainly absorbed by intestinal enterocytes when incorporated into these small micelles together with the rest of lipid compounds. They are formed during digestion when pancreatic lipases, bile acids, lecithin and other molecules transform the dietary fat into oily drops and large vesicles; the size of these oily drops and large vesicles is reduced until micelles are formed, generating a particular emulsion, and only the micelles with optimal dimensions are incorporated into the cell membranes and thus called DMMs. The larger-sized vesicles are usually excreted together with the precipitated compounds eliminated from the micelles. The lipids provided by the DMMs are only assimilated by enterocytes when specific transporters recognize them and transfer them to the endoplasmic reticulum. Apparently, for cholesterol absorption, the Niemann-Pick C1-like 1 protein is necessary, which shows high affinity for this molecule; thus, non-cholesterol sterols are incorporated in very low amounts as compared to cholesterol (approx. 2–5% vs. 60%).^{5,6}

The bioavailability of fungal sterols, such as ergosterol, could be enhanced by loading them onto specific micro-

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emulsions, improving, for instance, their anti-tumor properties.⁷ Microemulsions are thermodynamically stable nanometer systems used to stabilize compounds that are difficult to solubilize in physiological fluids. In addition to acting as cholesterol displacers in the DMMs, plant or fungal sterols have been described as modulators of the genes (SREBP, NR1H3 (LXR) DFDT1 (SQS), *etc.*) involved in the cholesterol metabolism, inhibitors of specific enterocyte enzymes needed for cholesterol absorption and transport (SOAT, MTT, APO48 *etc.*), liver X receptor (LXR) agonists, *etc.*^{8–10} However, at present, it is still unclear whether plant/fungal sterols need to be absorbed at higher concentrations to exert their hypocholesterolemic activities since when they are taken in large amounts, they also induce disorders such as sitosterolemia.¹¹ Moreover, if they are loaded onto microemulsions, they may have more difficulties to be incorporated into the DMMs and hence to displace cholesterol.

On the other hand, the inclusion of fungal β -glucan extracts into ergosterol-supplemented food matrices appeared to improve the cholesterol displacement from DMMs.⁴ These polymers also show hypocholesterolemic activities although their mechanism of action appears to be different from those postulated for sterols. These polysaccharides show gel forming properties, and they may increase viscosity during digestion, stimulating cholesterol excretion through faeces. They have also been reported as bile acid scavengers, enhancing the transformation of cholesterol to reestablish its levels. Moreover, the fermentation of β -glucans by colonic microbiota appears to stimulate the production of short-chain fatty acids that may block cholesterol biosynthesis by inhibiting HMGCR, a key enzyme in the cholesterol pathway.¹²

Ergosterol-enriched extracts can be successfully obtained from natural sources, such as mushrooms *e.g.* *Lentinula edodes*,^{13,14} *Agaricus bis porus*¹⁵ or *Agaricus brasiliensis*, using supercritical CO₂ extraction.¹⁶ The process is more effective than the use of organic solvents or other advanced technologies such as pressurized liquid-extraction or microwave/ultrasound-assisted technologies.^{15,17,18} However, the SFE plants utilized in most of the previous studies are usually small or pilot plant scale where the extraction conditions can be very easily controlled but lower amounts of extract are obtained as compared to the plants using higher volume extraction cells. Hence, to obtain extracts in large quantities for animal or clinical trials, a large-scale SFE plant should be utilized and some adjustments should be taken into consideration.

In this study, a large-scale SFE plant was used to obtain sufficient amounts of ergosterol-enriched extract from *Lentinula edodes* to carry out *in vitro* and *in vivo* tests to study its hypocholesterolemic activities. The obtained extract was loaded onto a microemulsion and mixed with a fungal β -glucan-enriched extract to investigate whether the microemulsion and the fungal extract could enhance or interfere with the activity of the obtained extract as a cholesterol displacer from DMMs during an *in vitro* digestion model; moreover, two different mice models were utilized to evaluate the *in vivo* hypocholesterolemic effect of the SFE extract and the β -glucan-enriched extract.

2. Materials & methods

2.1. Biological material, standards and reagents

Powdered *Lentinula edodes* S. (Berkeley) fruiting bodies (particle size < 0.5 mm, moisture < 5%) were purchased from Glucanfeed S.L. (La Rioja, Spain) and stored in the dark at –20 °C until use. Commercially available lard (Iberian pork lard, 99.7 g per 100 g fat) was purchased from a local supermarket and maintained at 4 °C until use. All the experiments were performed from the same lot.

Solvents such as hexane (95%), chloroform (HPLC grade), and methanol (HPLC grade) were obtained from LAB-SCAN (Gliwice, Poland), and absolute ethanol was obtained from Panreac (Barcelona, Spain). Calcium chloride, hydrochloric acid (37%), pepsin (from porcine pancreatic mucosa), sodium hydroxide, sodium chloride, Trizma base, maleic acid, pancreatin (from porcine pancreas), L- α -phosphatidylcholine (lecithin), Sepharose® 4B, phenol, ascorbic acid, BHT (2,6-Di-*tert*-butyl-*p*-cresol), Kolliphor®EL, and cholesterol (96%) and ergosterol (95%) (used as standards) were purchased from Sigma-Aldrich Química (Madrid, Spain). Carbon dioxide (99.99% purity) was supplied by Air-Liquid España, S.A. (Madrid, Spain).

2.2. Supercritical fluid extraction (SFE)

Extractions with CO₂ were carried out in a large scale SFE plant (Zean Consultores S.L., Madrid, Spain) comprising a 6 L cylinder extraction vessel and two different separators (S1 and S2) of 1.6 L capacity each with independent control of temperature and pressure. A detailed explanation of the experimental device can be found in the literature.¹⁹ The CO₂ flow rate was set at 20 L h^{–1}, and during the total extraction time tested herein (from 1 to 5 h), CO₂ was recirculated. The temperature was fixed at 40 °C in the extraction vessel and the separators, and the extraction pressure was maintained at 225 bar. Ethanol was not used as a co-solvent because previous studies had indicated that although higher yields could be obtained, the use of only CO₂ was more selective in generating fractions with higher sterol concentrations.¹⁵ The fractions extracted in 2 separators (S1 and S2) were obtained with ethanol and immediately submitted to drying using a rotary vacuum evaporator. Ethanol was utilized based on previous studies¹⁹ due to its following advantages: it is an organic solvent and easily evaporates *via* mild processes and it can completely solubilize the separated fractions by detaching the extracts from the separator walls. The dried extracts were stored at –20 °C until further analysis.

2.3. Preparation of ergosterol and SFE extract microemulsions

Sterol-loaded microemulsions were formulated as indicated by Yi *et al.* (2012)⁷ with slight modifications. Briefly, ergosterol (50 mg) or a selected SFE extract containing 52.5% ergosterol (SFE) (95 mg) was mixed with lard (1 g). Subsequently, Kolliphor EL (21% w/w) and 5% ethanol were mixed thoroughly and stirred as a surfactant-cosurfactant solution

followed by mixing with 3% of the supplemented lards. Then, water (71%) was added dropwise to the oily mixtures under gentle stirring to generate microemulsions: MERG (microemulsified ergosterol) and MSFE (microemulsified SFE extract) were prepared and immediately used in the *in vitro* digestion model.

2.4. Preparation of a β -glucan-enriched (BGE) extract

A β -glucan-enriched extract (BGE) was prepared by mixing different polysaccharide fractions, such as a fraction (2.5%) containing water-soluble β -glucans (named ExA by Morales *et al.* (2018)),²⁰ a fraction (26%) extracted with hot water (98 °C), filtered through a multichannel ceramic membrane (Ceramem Corporation, Waltham, USA) and concentrated with a spiral wound Nanomax50 membrane (Millipore, Bedford, USA) (called RF2 by Morales *et al.* (2019)²¹) and the remaining fraction (71.5%) containing mainly chitins and insoluble β -glucans, with hypocholesterolemic activities.²¹ The fractions were lyophilized, pooled together and stored at -20 °C until further use. The resulting mixture contained 33.5% (w/w dry weight) β -glucans and 0.23% ergosterol (determined by Morales *et al.* (2018)²⁰).

2.5. *In vitro* digestion and isolation of the dietary mixed micelles (DMMs)

Lard supplemented with cholesterol (25 mg g⁻¹) was used as a food matrix to evaluate the hypocholesterolemic activity of the different extracts in an *in vitro* digestion model, as reported by Gil-Ramirez *et al.* (2014).⁴ The hypercholesterolemic lard was mixed with ergosterol (ERG) (50 mg g⁻¹), MERG (32.3 g g⁻¹), SFE (95 mg g⁻¹) or MSFE (32.3 g g⁻¹) as ergosterol-containing formulations and/or BGE extract (149 mg g⁻¹) as a β -glucan plus ergosterol-containing extract (the indicated concentrations were adjusted to include 50 mg g⁻¹ ergosterol and/or β -glucans in the food matrix). The mixtures were gently stirred at their melting temperatures until complete incorporation of the supplements into the lipidic matrices occurred.

The supplemented food matrices (1 g) were submitted to *in vitro* digestion following the procedure described by Gil-Ramirez *et al.* (2014).⁴ After this, the fraction containing the dietary mixed micelles (DMMs) was isolated using a Sepharose®4B column with 0.15 M NaCl/16 mM bile salts as the mobile phase, as indicated by their intermicellar bile salt concentration (IMBC). The DMM fractions (16 mL) were identified because their cholesterol fraction (determined by an enzymatic SpinReact cholesterol quantification kit (SpinReact SAU, Girona, Spain) co-eluted with their phospholipid fraction (determined using an enzymatic Wako kit (Wako, Madrid, Spain)) at a proper elution volume (Gil-Ramirez *et al.*, 2014).⁴

2.6. Sterol quantification by GC-MS-FID

Sterols were extracted from the samples and quantified following the procedure described by Gil-Ramirez *et al.* (2013).¹⁵ The unsaponified fractions were injected into the Agilent 19091S-433 capillary column (30 m \times 0.25 mm ID and 0.25 μ m phase thickness). The column was connected to a 7890A

System gas chromatograph (Agilent Technologies, Santa Clara, USA) including a G4513A auto injector and a 5975C triple-axis mass spectrometer detector. The injector and detector conditions as well as the column temperature program were same as those described by Gil-Ramirez *et al.* (2013).¹⁵ Cholesterol and ergosterol were used as standards, and hexadecane (10% v/v) was used as an internal standard.

The GC-MS database identified the obtained peaks based on previous studies.^{15,22,23} The major detected sterols were cholesterol (RT = 11.9 min), ergosterol (ergosta-5,7,22-trien-3 β -ol) (RT = 12.6 min), ergosta 7,22-dienol (RT = 12.8 min), ergosta-5,7-dienol (RT = 13.1 min) and ergosta-7-enol (fungis-terol) (RT = 13.3 min).

2.7. Animal trials

The male mice C57/BL6Jrj (6 weeks old; Janvier SAS, Le Genest-Saint-Isle, France) were maintained under the following temperature-, humidity- and light-controlled conditions: 24 \pm 2 °C, 40–60% humidity, and 12:12 hours light:dark cycle, respectively; moreover, they had free access to water and food. In addition, two different experiments were carried out, and both were approved by the Institutional Animal Welfare and Ethics Committee of La Paz University Hospital (Madrid, Spain) according to the current Spanish and European legislation (RD53/2013 and EU 63/2010).

In the first experiment, 35 mice were randomly divided into four groups. The group NC (normal control, n = 5) was fed a control diet (standard diet, Safe Rodent Diet, A04, Augy, France), the group HC1 (high cholesterol, n = 10) was administered a high fat hypercholesterolemic diet (HFHD) (standard diet supplemented with 1.25% cholesterol, 0.5% cholic acid and 12% lard), the group ERG (n = 10) was administered the HFHD supplemented with ergosterol (0.45%) and the group SFE (n = 10) was fed HFHD supplemented with the SFE extract (up to 0.45% ergosterol in the diet). Blood samples were obtained by mandibular puncture at the beginning and the end of the study (5 weeks), and plasma samples were stored at -20 °C until further use.

In the second experiment, 20 mice were fed the standard diet supplemented with 1.25% cholesterol and 0.5% cholic acid, and the blood samples were obtained at the beginning and 3 weeks after the feeding started. After 4 weeks, the animals were randomly divided into two groups (n = 10): one group was administered the same diet and used as a control (HC2), and the other (BGE) was administered the diet supplemented with 6% BGE. Both were fed the diet during 5 weeks.

After the feeding periods, mice were obtained from both experimental groups and killed by intracardiac exsanguination under anesthesia with 1.5% isoflurane; moreover, plasma was obtained and stored at -20 °C before use. Livers, fat and kidneys were removed, immediately frozen in liquid nitrogen and stored at -80 °C. The levels of total cholesterol, triglycerides, HDL-cholesterol and LDL-cholesterol in the plasma were measured using the Cobas C311 Autoanalyzer (Roche, Spain).

2.8. Statistical analysis

Differences were evaluated at a 95% confidence level ($P \leq 0.05$) using one-way analysis of variance (ANOVA) followed by the Tukey's Multiple Comparison test. Statistical analysis was performed using the SPSS V.13.0 software (SPSS Institute Inc., Cary, NC) and GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA, USA).

3. Results and discussion

3.1. Upscaling of the SFE extraction

Large-scale supercritical CO₂ extractions were carried out to scale up the production of the SFE extracts from shiitake mushrooms since similar extracts obtained from *Agaricus bis porus* decreased the TC/HDL ratio in hypercholesterolemic mice when applied at low concentrations. In this regard, Gil-Ramirez *et al.* (2016)⁹ have concluded that to improve their hypocholesterolemic activity, the SFE extracts should be utilized at higher concentrations for *in vivo* testing.

The extraction yields increased with an increase in the extraction time up to 3 h, reaching the value of 0.53% taking into consideration the material obtained in both separators (S1 + S2) (Fig. 1). However, when longer extraction times were used (up to 5 h), only a slight increase in the extraction yield was noticed (0.56% yield) after the additional 2 h extraction period. The S1 separator collected more material than the S2 separator (2.3–2.8 fold) regardless of the extraction time. Previous studies reported on the extractions from shiitake mushrooms using a pilot scale plant under similar conditions (225 bar, 35 °C, and 3 h) showed higher yields (1.25%).¹⁴ These yields could be improved in pilot-plants by increasing the temperature (2.3% at 225 bar and 75 °C) or pressure (2.1% at 350 bar and 40 °C). However, other researchers obtained similar values in the shiitake SFE at 40 °C during 3 h of extraction under 200 and 250 bar (0.65 and 0.81%, respectively) using a smaller plant with a 100 mL extraction cell.¹³

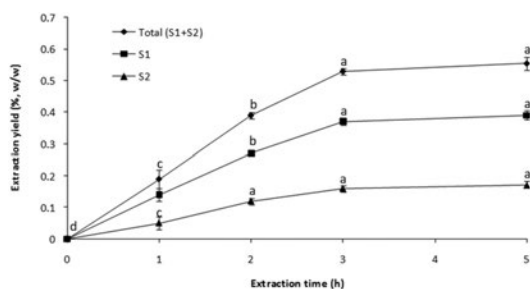


Fig. 1 SFE yields obtained in the separator 1 (S1), separator 2 (S2) and both separators (S1 + S2) at different extraction times. ^{a–d}Different letters denote significant differences ($P < 0.05$) between different extraction times.

The obtained SFE extracts contained up to 53% ergosterol (Table 1), similar to the value indicated by Gil-Ramirez *et al.* (2013)¹⁵ for *Agaricus bis porus* but significantly higher than the others noticed for *L. edodes* using a smaller capacity pilot plant.¹⁴ The results showed that although the yield in this large-scale plant was lower, the extraction was apparently more selective towards sterols than the case when a pilot plant was used. Moreover, when the ergosterol content was determined in the extracts collected in both separators, the S1 extracts exhibited higher cholesterol concentrations (41–53%) than the S2 extracts (19–26%), and statistically significant differences were found in S1 between 1 and 5 h of extraction time, suggesting that time could influence the ergosterol enrichment of the extracts.

Ergosterol derivatives were also detected in the extracts, but at lower concentrations (Table 1). The concentration of fungisterol was higher than those of ergosta5,7-dienol and ergosta7,22-dienol, and it was distributed within the separators in a similar ratio to ergosterol. However, ergosta7,22-dienol was specifically recovered in S2, and the concentration of ergosta5,7-dienol showed no differences between separators. Thus, the extract containing a larger amount of fungal sterols (58.7%) obtained after 5 h in S1 was selected as the SFE extract to carry out further experiments.

3.2. *In vitro* testing of the hypocholesterolemic properties of the obtained extracts

Previous *in vitro* studies indicated that ergosterol and, in particular, an SFE extract containing fungal sterols obtained from *A. bis porus* were as effective as β -sitosterol in the displacement of cholesterol from DMMs when they were incorporated in a food matrix such as lard.⁴ To test the hypocholesterolemic ability of the SFE extract obtained from *L. edodes*, the same *in vitro* digestion model was reproduced. The results indicated that the DMM fraction generated after the digestion of the lard supplemented with cholesterol and ergosterol (ERG) contained approx. 63% less cholesterol than the case when only cholesterol was added (Fig. 2). This reduction was slightly higher than the 49% reduction noticed by Gil-Ramirez *et al.* (2014);⁴ however, when the SFE extracts were added, this effect was almost the same (69% and 67%); this indicated that regardless of the mushroom utilized, the SFE extracts were able to reduce the cholesterol levels in the DMM fraction (the fraction usually absorbed by intestinal enterocytes).

Moreover, according to previous studies, the bioavailability of phytosterols as well as other non-cholesterol sterols is very limited;⁸ however, other studies have indicated that ergosterol bioavailability may be enhanced by loading the extracts onto microemulsions;⁷ on the other hand, if sterols are incorporated into artificial vesicles followed by their incorporation into food matrices, they may be more bioavailable but perhaps would not be able to act as cholesterol displacers in the DMMs. Therefore, ergosterol and the SFE extract were emulsified (MERG and MSFE, respectively), supplemented to the hypercholesterolemic lard and digested to investigate the content of the generated DMM fractions. The results indicated

Table 1 Fungal sterol contents in the SFE extracts recovered in the separator 1 (S1) and separator 2 (S2) at different extraction times (1, 2, 3 and 5 h). n.d. = not detected; ^{a-c}Different letters denote significant differences ($P < 0.05$) between different extraction times for the same sterol

Extraction time (h)	Ergosterol (%)		Ergosta7,22-dienol (%)		Ergosta5,7-dienol (%)		Fungisterol (%)	
	S1	S2	S1	S2	S1	S2	S1	S2
1	41.44 ± 3.40 ^b	21.52 ± 2.31 ^c	n.d. ^b	1.19 ± 0.28 ^a	0.61 ± 0.13 ^{ab}	0.22 ± 0.05 ^b	4.15 ± 0.19 ^b	2.31 ± 0.23 ^c
2	46.30 ± 1.49 ^{ab}	20.30 ± 0.33 ^c	n.d. ^b	1.04 ± 0.23 ^a	0.48 ± 0.02 ^{ab}	0.16 ± 0.03 ^b	3.83 ± 0.05 ^{bc}	1.83 ± 0.45 ^c
3	46.12 ± 2.06 ^{ab}	18.56 ± 1.03 ^c	n.d. ^b	1.08 ± 0.19 ^a	0.46 ± 0.07 ^{ab}	0.40 ± 0.11 ^b	3.92 ± 0.13 ^{bc}	2.76 ± 0.49 ^c
5	52.50 ± 1.76 ^a	25.90 ± 0.18 ^c	n.d. ^b	1.54 ± 0.07 ^a	0.71 ± 0.09 ^a	0.65 ± 0.02 ^{ab}	5.50 ± 0.36 ^a	3.79 ± 0.09 ^{bc}

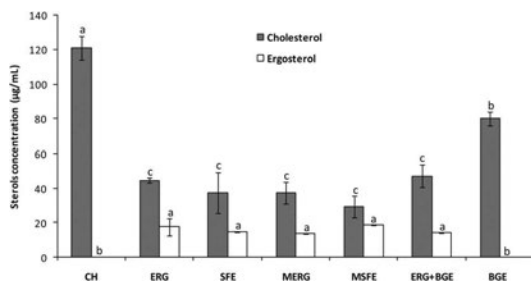


Fig. 2 Cholesterol and ergosterol concentrations in the isolated DMM fractions generated after *in vitro* digestion of the lard supplemented with cholesterol (CH) and cholesterol together with ergosterol (ERG), SFE extract (SFE), microemulsified ergosterol (MERG), microemulsified SFE extract (MSFE), ergosterol + β -glucan-enriched extract (ERG + BGE) and only β -glucan-enriched extract (BGE). ^{a-c}Different letters denote significant differences ($P < 0.05$) between different samples for the same compound.

that both extracts could displace cholesterol from the generated DMMs as their corresponding non-microemulsified extracts.

Other studies have suggested that the presence of β -glucan extracts obtained from *Pleurotus ostreatus* in the food matrix functionalized with ergosterol reduces approx. 52% more cholesterol content of the generated DMMs than the case when only the sterol is added.⁴ When similar food mixture was prepared using a β -glucan-enriched extract obtained from *L. edodes* (BGE + ERG), a lower cholesterol level was noticed in the generated DMM fraction than that in the DMM control (CH); however, no significantly different values were noted as compared to the case of the DMM fraction generated when the food matrix was supplemented only with ERG. Differences with previous studies might be due to the structural differences between the polysaccharides obtained from both mushroom species or because of their specific extract compositions. Surprisingly, when the BGE extract was also tested without any sterol addition, a significant cholesterol reduction was noticed (33.7%) in its DMM fraction as compared to that in the control. Since the ergosterol levels in the BGE extract were really low (0.23%), the noticed hypocholesterolemic activity might be because of its high β -glucan content (33.5%). These polymers were able to scavenge other small molecules, such as

bile acids or cholesterol, in their structures.^{24,25} In fact, it is suggested as their potential mechanism for lowering the cholesterol levels in serum¹² although other mechanisms may be involved.²⁶

Ergosterol was also detected in the generated DMM fractions, but it was always incorporated in lower concentrations than cholesterol (on average 58% less); this was in agreement with the previous results;⁴ moreover, no significant differences were noticed between the different tested extracts; this suggested that the incorporation of ergosterol in the DMM had a significant effect on the ergosterol content in the food matrix than on the presence of other components (such as β -glucans and surfactants) in the mixture because all the extracts were supplemented to the hypercholesterolemic lard at different concentrations to ensure the achievement of the same ergosterol content.

3.3. *In vivo* testing of the hypocholesterolemic properties of the obtained extracts

Animal studies were carried out to confirm the hypocholesterolemic activity noticed *in vitro* for the extracts. However, to minimize the number of mice utilized for this experiment, only ergosterol as a standard compound and the SFE extract were tested since no significant differences were found with the MERG or MSFE in terms of their capacity to displace cholesterol from DMMs. The MSFE preparation included more processing that might increase the cost if up scaled. Moreover, the administration of the preparations and the induction of hypercholesterolemia were simultaneously carried out to study the potential of these preparations as preventive more than palliative formulations since the latter influence was already investigated for a SFE extract obtained from *A. bis porus*.⁹

The mice groups treated with normal diet (NC) or hypercholesterolemic diet (HC1) used as a control as well as the ERG and SFE groups showed similar values of total cholesterol (TC) (91.4 ± 10.8 mg dL⁻¹ on an average), HDL (1.9 ± 0.7 mmol L⁻¹), LDL (0.2 ± 0.1 mmol L⁻¹), triglycerides (TG) (158 ± 68.8 mg dL⁻¹), AST (74.8 ± 36.1 UI dL⁻¹), ALT (18.0 ± 8.1 UI dL⁻¹) and glucose (50.0 ± 27.5 mg dL⁻¹) at the beginning of the experimental period. However, after 5 weeks, only the NC group maintained these levels, and the rest of the groups exhibited increased TC levels with non-significant differences between HC1 (230.3 ± 67.1 mg dL⁻¹), ERG (277.3 ± 35.5 mg dL⁻¹) or SFE (262 ± 52.6 mg dL⁻¹) groups. The increase noticed in the

ERG group was more influenced by a high LDL value (5.7 ± 1.0 , whereas HC1 was 4.6 ± 1.7 mmol L⁻¹) and that noticed in the SFE group was more influenced by a high HDL value (3.2 ± 1.0 , whereas HC1 was 2.2 ± 0.3 mmol L⁻¹); however, differences in both parameters were insignificant. Similarly, the TC and glucose values increased in all the groups (from 40.5 to 45.6 mg dL⁻¹ and from 155.5 to 192.7 mg dL⁻¹, respectively). Therefore, the ergosterol-containing preparations showed no hypocholesterolemic activity *in vivo*. These results differ from those reported in the recent studies carried out in rats fed simultaneously a hypercholesterolemic diet supplemented with 0.5% or 1.5% ergosterol for 8 weeks²⁷ or previous tests carried out in mice where first hypercholesterolemia was induced followed by treatment with ergosterol-containing preparations obtained from *A. bis porus*.⁹

Thus, since the BGE extracts also showed hypocholesterolemic activity *in vitro* but their mechanism of action might be different from that of fungal sterols, another *in vivo* experiment was carried out to investigate whether the β -glucan-enriched extract was more effective than the fungal sterols. This time, the experimental setting was changed; moreover, at first, the mice were fed a hypercholesterolemic diet, and then, they were administrated the BGE extract together with unhealthy diet to investigate the effect of the extracts as palliative compounds. The results indicated that the administration of the standard diet supplemented with cholesterol and cholic acid for 3 weeks increased the TC, HDL and LDL levels in all the mice, inducing hypercholesterolemia (Table 2). The glucose concentration and hepatic enzyme activities were also influenced, and their values were increased. Only the TG amounts remained unchanged. After 4 weeks, the HC2 group continued with the hypercholesterolemic diet, and the BGE group diet was supplemented with the BGE extract for 5 weeks. After this feeding period, the BGE group exhibited significantly lowered TC values, more than 1.5 fold lower than the HC2 group levels. The reduction might be due to the lower HDL and LDL levels noticed in the BGE group. The different diet did not modify the other determined parameters since the TG, AST, ALT and glucose values were similar in both groups. These results were in line with previous findings where mice fed with lard supplemented with a β -glucan extract obtained from *P. ostreatus* prevented the TC increase induced by a hypercholesterolemic diet administrated during 5 weeks.²⁸ Similar hypocholesterolemic activities were noticed for *A. bis*

porus, *Grifola frondosa*, *Flammulina velutipes* and *L. edodes* dietary fibers but on normocholesterolemic rats.^{29,30} However, in other studies where a β -glucan-enriched extract (from *P. ostreatus*) was administrated, the BGE (as palliative compounds) did not significantly reduced the TC levels in mice serum.³¹ The different mushroom species or the administration of β -glucans at higher concentrations than those utilized in the study might be the reason for the higher effectiveness of the BGE extract obtained from *L. edodes*.

4. Conclusions

Herein, ergosterol-enriched extracts were successfully obtained from *Lentinula edodes* fruiting bodies by supercritical CO₂ extraction with levels up to almost 53% ergosterol (approx. 59% of total sterols). The extraction yields almost linearly increased with an increase in the extraction time up to 3 h; however, the extracts obtained after 5 h contained higher sterol concentrations. The extracts collected in the separators showed slightly different compositions: the S1 extracts had higher ergosterol and fungisterol contents than the S2 extracts. However, ergosta7,22-dienol was exclusively found in S2. The SFE extract collected after 5 h in S1 and commercial ergosterol were also microemulsified and tested as cholesterol displacers using an *in vitro* digestion model where the DMM fraction was isolated. Ergosterol and the SFE extract with or without microemulsion succeeded to reduce the cholesterol levels within the generated DMMs, showing potential as hypocholesterolemic compounds. However, when they were administrated concomitantly with a hypercholesterolemic diet to a mice model, no reduction in the serum cholesterol levels was noticed as compared to the case of the control. Perhaps the hypocholesterolemic effect reported for other SFE extracts was only effective when the extracts were administrated as palliative ingredients. These results indicate that although *in vitro* testing may be encouraged to screen the biological activities of many bioactive compounds, *in vivo* studies should also be performed as the results may disagree with the *in vitro* results. For instance, the BGE extract obtained from *L. edodes* with a low ergosterol content (0.23%) but a high β -glucan concentration (33.5%) showed lower ability to displace cholesterol in the *in vitro* model but it was able to successfully reduce the cholesterol levels in the hypercholesterolemic mice model. Therefore,

Table 2 Total (TC), HDL (HDL) and LDL-cholesterol (LDL), triglycerides (TG), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and glucose (GLC) values of the mice fed 9 weeks with HFHD (HC2) and HFHD supplemented with BGE from week 4 to 9. Blood samples were obtained after 0, 3 and 9 weeks of administration. Asterisks indicate statistically significant differences between the control and BGE group (**P* < 0.05; ***P* < 0.001)

Time (weeks)	Group	TC (mg dL ⁻¹)	HDL (mmol L ⁻¹)	LDL (mmol L ⁻¹)	TG (mg dL ⁻¹)	AST (UI dL ⁻¹)	ALT (UI dL ⁻¹)	GLC (mg dL ⁻¹)
0	HC2	43.05 ± 8.96	1.01 ± 0.17	0.15 ± 0.03	69.26 ± 19.67	44.49 ± 17.19	10.01 ± 2.47	29.78 ± 12.12
	BGE	34.53 ± 14.35	0.81 ± 0.24*	1.51 ± 2.89	54.83 ± 23.95	47.39 ± 28.26	8.53 ± 4.71	20.43 ± 9.50
3	HC2	96.68 ± 32.72	1.18 ± 0.22	2.06 ± 0.77	30.03 ± 8.12	113.89 ± 57.61	122.56 ± 120.53	56.08 ± 13.98
	BGE	97.11 ± 33.03	1.17 ± 0.22	2.22 ± 0.84	24.78 ± 9.92	71.26 ± 47.39	95.71 ± 130.31	66.78 ± 17.65
9	HC2	259.58 ± 48.26	2.01 ± 0.13	5.60 ± 1.45	34.68 ± 5.66	119.41 ± 61.69	92.61 ± 60.94	64.69 ± 18.57
	BGE	162.51 ± 33.78**	1.62 ± 0.31*	3.62 ± 0.52*	29.97 ± 3.98	154.61 ± 62.64	146.53 ± 74.06	74.70 ± 27.24

further investigation is encouraged to test BGE hypocholesterolemic activity. Thus, clinical trials must be carried out before its utilization in novel functional food formulations.

Abbreviations

ERG	Ergosterol
SFE	Supercritical fluid extraction
DMM	Dietary mixed micelle
TC	Total cholesterol
NC	Normal control
HFHD	High fat high hypercholesteromic diet
HC	High cholesterol
BGE	β -Glucan-enriched extract
MERG	Microemulsified ergosterol
MSFE	Microemulsified supercritical fluid extract
TG	Triglycerides
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
GLC	Glucose

Conflicts of interest

There are no conflicts to declare.

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Effect of traditional and modern culinary processing, bioaccessibility, biosafety and bioavailability of eritadenine, a hypocholesterolemic compound from edible mushrooms

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Eritadenine is a hypocholesterolemic compound that is found in several mushroom species such as *Lentinula edodes*, *Marasmius oreades*, and *Amanita caesarea* (1.4, 0.7 and 0.6 mg per g dry weight, respectively). It was synthesized during all developmental stages, being present in higher concentrations in the skin of shiitake fruiting bodies. When subjected to traditional cooking, grilling followed by frying were more adequate methodologies than boiling or microwaving to maintain its levels. Modern culinary processes such as texturization (with agar-agar) and spherification (with alginate) also interfered with its release. Grilling and gelling using gelatin enhanced eritadenine's bioaccessibility in an *in vitro* digestion model. An animal model (where male and female rats were administered 21 and 10 mg per kg animal per day of eritadenine) indicated that intake of the compound was safe under these concentrations; it reached the liver and reduced the atherogenic index (TC/HDL) in rat sera. Thus, it might be used to design a functional food.

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1. Introduction

Edible mushrooms are consumed worldwide due to their particular flavors and nutritional values. Their consumption is also encouraged because of their health promoting properties *e.g.* their ability to lower cholesterol levels in serum. Therefore, certain mushroom species could be used as starting material to design functional foods. They contain fungal sterols and β -glucans that can impair dietary cholesterol absorption¹ and other molecules that affect the biosynthesis of endogenous cholesterol.^{2,3} They can also be used to synthesize other compounds such as eritadenine that can also indirectly influence serum LDL levels.⁴

Eritadenine ((2*R*),3*R*)-dihydroxy-4-(9-adenyl)butanoic acid), also named lentysine or lentynacin, is an adenosine analog derived from secondary metabolism that was firstly iso-

lated from shiitake mushrooms (*Lentinula edodes*).⁵ Several studies demonstrated that its hypocholesterolemic activity in mice and rats was related to its ability as an *S*-adenosyl-L-homocysteine hydrolase (SAHH) inhibitor. Eritadenine modulated hepatic phospholipid metabolism by decreasing the phosphatidylcholine (PC)/phosphatidylethanolamine (PE) ratio. However, its precise mechanism of action has not yet been fully elucidated^{4,6,7} and therefore certain safety risks might arise if food products are functionalized by adding eritadenine-enriched extracts as hypocholesterolemic ingredients.

Another aspect to take into consideration is the fact that edible mushrooms (or their functionalized foods) are not usually consumed raw but they are subjected to culinary treatments before ingestion. These processes, usually involving heat (boiling, grilling, *etc.*), might modulate the concentrations of the fungal bioactive molecules that are assimilated.^{8,9} Moreover, novel culinary procedures that are nowadays in trend, presenting surprising dishes or food products (gelling, spherification, *etc.*) to potentiate consumer interest, might influence their levels too since they include food additives in their formulations (hydrocolloids, thickeners, *etc.*). These additives could directly interact with the bioactive ingredients, modifying their bioaccessibility. Later on, the bioactive compounds that survive the culinary treatments might be further modified during mastication, stomach and/or intesti-

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nal digestions influencing their absorption levels at the human intestine.¹⁰ However, not many studies have been carried out to evaluate the influence of all these processes on bioactive compounds such as eritadenine.

Therefore, a screening through several edible mushrooms was carried out to find an interesting eritadenine source. Different fruiting body tissues and developmental stages were also investigated. Then, shiitake mushrooms were subjected to traditional and modern culinary processing to evaluate their effect on eritadenine stability. Afterwards, the effect of digestion on eritadenine bioaccessibility was studied using an *in vitro* digestion model and its toxicity, bioavailability and hypocholesterolemic effect were evaluated with *in vivo* animal experiments.

2. Materials & methods

2.1. Biological material

Mushroom species such as *Lentinula edodes* S. (Berkeley), *Lactarius deliciosus* (Fr.), *Boletus edulis* (Bull. Ex Fr.), *Pleurotus ostreatus* (Jacq. Ex Fr.) Kummer, *Agaricus bisporus* L. (Imbach), *Amanita caesarea* (Scop. Ex Fri.) Pers. Ex Schw., *Cantharellus tubaeformis* (Schaeff) Quel, *Ganoderma lucidum* (Curtis) P. Karst., *Lyophyllum shineji* (Kawam.), *Morchella conica* (Pers.), *Auricularia auricula judae* (Bull. Ex St. Amans) Berck, and *Marasmius oreades* (Bolt. Ex Fr.) Fr were purchased in season from the local market in Madrid (Spain). They were lyophilized and ground into fine powder as described by Ramirez-Anguiano *et al.* (2007).¹¹ Dried mushroom powders were stored at -20°C until further use.

Fresh *Lentinula edodes* fruiting bodies were also subjected to traditional cooking treatments (see later) or processed to separate the different tissues with a knife. Then, individual parts were treated as described above.

In order to test an eritadenine-enriched preparation as a potential functional ingredient for the *in vivo* tests, an extract was obtained by stirring *L. edodes* powder with water (0.5 g L^{-1}) for 1 min at room temperature. The resulting suspension was centrifuged for 7 min at 7000 rpm at 10°C and the obtained supernatant was immediately frozen and lyophilized. This fraction was an eritadenine-enriched extract containing 3.1 mg g^{-1} .

2.2. Reagents

Solvents such as methanol (HPLC grade) and acetonitrile (HPLC grade) were acquired from Lab-Scan (Gliwice, Poland). Ethanol (HPLC grade), diethyl ether (HPLC grade) and calcium chloride were purchased from Panreac (Barcelona, Spain). Hydrochloric acid (37%), trifluoroacetic acid (99%), sodium hydroxide, pepsin (from porcine gastric mucosa), sodium chloride, Trizma base, maleic acid, pancreatin (from porcine pancreas) and 1- α -phosphatidyl choline (lecithin) were obtained from Sigma-Aldrich (Madrid, Spain) and D-eritadenine (90%) from SYNCHEM UG & Co. KG (Felsberg, Germany).

All additives used for culinary preparations were food grade: gelatin was acquired from Mondelez International (Madrid, Spain) and agar-agar (E406), sodium alginate (E401) and calcium lactate (E327) from Cuisine Innovation (Dijon, France).

2.3. Traditional culinary processing

Lentinula edodes fresh fruiting bodies were cut into slices and cooked (30 g) following four traditional methods: grilling, microwave cooking, frying and boiling. These treatments were carried out as described by Soler-Rivas *et al.* (2009)⁹ in quadruplicate and the resulting cooked mushrooms were directly subjected to an *in vitro* digestion model or freeze-dried, ground and stored at -20°C until further analysis.

2.4. Modern culinary processing

Lyophilized shiitake fruiting bodies were subjected to modern culinary processes used on molecular gastronomy such as thickening (usually called 'texturization'), gelling and 'spherification'.

Powdered shiitake mushrooms (500 mg) were mixed with 50 mL water and 1 g agar-agar (as a thickener) and homogenized with a culinary blender (Minipimer 3MR320 Braun, Aschaffenburg, Germany). Afterwards, the mixture was heated up to 100°C and maintained for 1 min. Then, an aliquot was collected with a 60 mL syringe and injected into a silicone tube (77 cm length and 0.5 cm diameter). The filled tube was partially folded, making loops, and submerged in an ice bath to cool down. The semi-solidified (texturized) gel obtained was extracted from the silicone tube by forcing air inside with another syringe. Then, the texturized shiitake powder resembled semi-transparent spaghetti with a mushroom taste.

Powder from the same shiitake batch (500 mg) was added to a previously hydrated gelatin solution obtained by dissolving a gelatin film (1 g) in cold water (50 mL) for 3 min. Then, the mixture was stirred at 75°C for 2 min. Afterwards, the homogenate was poured into a round mold and stored at 4°C for 2 h to allow gel formation.

'Spherification' was carried out by mixing the shiitake powder (500 mg) and sodium alginate (400 mg) with 50 mL water and homogenizing them with a blender until a slightly viscose solution was obtained. The mixture was kept for 5 min at room temperature to eliminate bubbles. Then, it was introduced into a 60 mL syringe and slowly dropped on a calcium lactate solution (44 mM). The surface of the liquid drops polymerized when placed in contact with Ca^{2+} , yielding jelly spheres. Spheres were left for approx. 3 min, transferred to a water bath to remove calcium excess, taken out with a perforated spoon and plated.

Each culinary process was carried out 4 times. The resulting preparations were directly subjected to an *in vitro* digestion model or freeze-dried, ground and stored at -20°C until further analysis.

2.5. *In vitro* digestion model

Fresh shiitake fruiting bodies, as well as the food preparations resulting from the traditional and modern culinary processes, were digested following the procedure described by Gil-Ramirez *et al.* (2014)¹ with modifications. Samples (15 g of traditional culinary processed shiitake or the modern preparations including 500 mg shiitake powder) were masticated by a volunteer for approx. 2 min and spat into a beaker. Milli-Q water was acidified with 6 M HCl (adjusting the pH to 2.0) and added (54 mL) to the masticated samples. The mixture was transferred to a thermostatic vessel at 37 °C with mild stirring and pepsin (275 mg) was also incorporated. Then, it was incubated for 1 h and stirred in a titrator device (Titrino plus, Metrohm, Herisau, Switzerland) simulating gastric digestion. Afterwards, intestinal digestion was initiated by adding 5 mM CaCl₂ and 150 mM NaCl and adjusting the pH to 6.0 by adding 0.5 M NaOH. Then, a pancreatic solution (6 mL) containing 20 mg pancreatin, 633 mg bile extract and 228 mg lecithin (in 50 mM Trizma-maleate buffer pH 7.5) was added and the pH was adjusted to 7.5 and maintained for 2 h using a viscotrode (Metrohm, Herisau, Switzerland) placed in the titrator device. The stirring level and temperature were the same as that used in the gastric digestion simulation.

After the digestion process, digested samples were heated in a water bath at 80 °C for 10 min to inactivate digestive enzymes (eritadenine was resistant to this thermal treatment as indicated elsewhere^{12,13}) and then subjected to centrifugation (7000 rpm for 15 min) to separate the supernatant that was considered as the bioaccessible fraction.

Eritadenine bioaccessibility was estimated to be the ratio (%) between the amount of eritadenine in the bioaccessible fraction and that in the sample before the digestion process.

2.6. Animals and diets

Sprague Dawley adult (5 weeks old) male and female rats ($n = 24$) were purchased from Charles River (San Cugat del Valles, Barcelona, Spain) and housed, separated by sex, in groups of four animals per cage. Animals were maintained under controlled conditions of temperature, humidity and light (24 ± 2 °C, 40–60% humidity, 12 h:12 h light/dark cycle) and had free access to water and food (commercial rodent maintenance diet A04; Scientific Animal Food & Engineering, Augy, France). After an adaptation period (6 days) animals were weighed and randomly divided into three groups per sex: control group (C) that remained with the standard diet, another group that was fed with a low eritadenine dose (10 mg per kg animal per day) (LE) and the third group was fed a higher dose (HE) (21 mg per kg animal per day). Diets were prepared mixing A04 chow with the corresponding amount of the eritadenine-enriched extract necessary to obtain the indicated eritadenine concentration per group. The nutrient composition of each diet is summarized in Table 1. Animals were maintained on this diet for 5 weeks with daily evaluation of behavioral (posture and activity) and physiological (fur and mucosa status, hydration and the presence of secretions and wounds) parameters were

Table 1 Nutritional composition of diets. Rodent standard diet (A04) supplemented with high and low doses of the eritadenine-enriched extract containing 3.1 mg g⁻¹ eritadenine. The results are expressed as g per 100 g in dry base

	High dose	Low dose	Control
Carbohydrates	54	57	60
Protein	14.4	15.3	16
Lipids	2.7	2.9	3
Dietary fibre	3.6	3.8	4
Eritadenine-enriched extract	10	4.8	—

scaled by trained staff with weekly monitoring of weight gain. Feces were collected at the beginning and at the end of the experimental period and maintained at -20 °C until further use.

The protocol was approved by the Institutional Animal Ethics Committee of La Paz University Hospital (Madrid, Spain) and procedures were performed in accordance with the EU Directive 2010/63/EU and the Spanish law RD 53/2013 regarding the protection of experimental animals.

2.7. Biosafety and bioavailability studies

Following the experimental feeding period (5 weeks), overnight-fasted rats were euthanized by intracardiac exsanguination under anesthesia with 1.5% isoflurane. Plasma was separated out by centrifugation (10 min at 5000 rpm) using sterile tubes pre-treated with EDTA. The supernatant collected was stored at -20 °C until analysis. Plasma levels of total cholesterol (TC), HDL cholesterol, triglycerides, glucose and circulating renal and liver damage biomarkers (creatinine, uric acid, bilirubin, alanine transaminase (ALT), aspartate transaminase (AST)) were measured in duplicate for each sample using a Covas C311 Autoanalyzer (Roche, Basel, Switzerland) specifically calibrated for rodent samples. All enzymatic colorimetric kits and internal quality controls were supplied by Roche (Basel, Switzerland).

Livers, spleen, kidneys and testis or ovaries were collected, weighed and washed in ice-cold PBS. Samples were processed for each tissue, keeping a fraction fixed in 10% neutral buffered formalin for 24 hours and the other part was immediately frozen in liquid nitrogen and stored at -80 °C until analysis. Fixed samples were embedded in paraffin for further analysis by immunohistochemistry. Afterwards, frozen liver samples were subjected to homogenization to detect eritadenine (see later).

2.8. Eritadenine determination by HPLC-DAD

Eritadenine was extracted from samples and quantified following two different methods. One of them (method 1) was based on the procedure of Enman *et al.* (2007)¹⁴ with slight modifications. In this case, samples (1 g) were mixed with 20 mL of 80% methanol (v/v) and stirred in the dark for 3 h. Then, the mixture was filtered through a 14–18 µm pore size paper filter (GE Healthcare Europe GmbH 1240, Barcelona, Spain) and methanol was removed on a rotary vacuum evaporator (60 °C),

keeping the sample protected from light. Afterwards, the dried extract was mixed with 10 mL Milli-Q water, washed 3 times with diethyl ether, mixed with 40 mL ethanol (4 : 1, v/v) and kept overnight at -20°C . Finally, the sample was filtered through filter paper, and ethanol was removed using the rotary vacuum evaporator and water using a freeze-dryer. The resulting eritadenine extract was stored at -20°C until further use.

The second extraction method (method 2) followed the Afrin *et al.* (2016)¹⁵ procedure. Briefly, samples (1 g) were mixed with 10 mL of 60% ethanol (v/v) and stirred for 2 min. The mixture was subjected to centrifugation (7000 rpm, 15 min) in a Heraeus Multifuge 3SR+ centrifuge (Thermo Fisher Scientific, Madrid, Spain) and the supernatant was carefully collected. The pellet was extracted twice and supernatants were pooled together, filtered and dried as explained in method 1.

Eritadenine was extracted from the livers by homogenizing the organs with an IKA Werke T8 Ultra Turrax (Ika Works Inc., Staufen, Germany) at maximum power. The resulting homogenates were mixed with 10 mL of 60% ethanol (v/v) and stirred for 2 min, following method 2.

The identification and quantification of eritadenine were carried out using an HPLC system (Pro-Star 330, Varian, Madrid, Spain) equipped with a PDA detector (Pro-Star 363 module, Varian, Madrid, Spain). Samples were dissolved in the mobile phase (5 mg mL⁻¹), injected (10 μL) into a C18 Spherisorb ODS2 analytical column (4 \times 250 mm, 5 μm , Waters, Missisagua, Ontario, Canada) and developed at 0.5 mL min⁻¹ with water : acetonitrile (98 : 2, 1% v/v TFA). Eritadenine was quantified at 260 nm using a commercial standard (0.004–0.25 mg mL⁻¹). The compound eluted after 10.5 min and showed the characteristic eritadenine UV-spectrum.

2.9. Statistical analysis

Differences were evaluated at 95% confidence level ($P \leq 0.05$) using one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison test. Statistical analysis was performed using SPSS V.13.0 software (SPSS Institute Inc., Cary, NC) and GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA, USA).

3. Results and discussion

3.1. Determination of eritadenine in several edible mushrooms

Eritadenine was first isolated from *Lentinula edodes*⁵ but not many other species were investigated to study whether they might contain interesting concentrations. Therefore, before selecting shiitake as a source of the metabolite, a preliminary screening of a few other related mushroom species was carried out. Eritadenine was extracted from selected mushrooms following two already described methods because they both claimed to be specific for eritadenine determination but their procedures were different.^{14,15} The results indicated that indeed *L. edodes* was the mushroom with higher eritadenine concentrations but a few other species contained it too

although in lower concentrations (Fig. 1a). Extraction of eritadenine using method 1 yielded slightly lower levels of this metabolite than extraction using method 2 and this difference seemed to occur similarly within all the analyzed species. Apparently, the longer incubation times utilized in method 1 did not increase the extraction yield.

Lentinula edodes contained 1.0 or 1.4 mg g⁻¹ (depending on the method utilized), levels that were lower than those in some publications^{14,15} but higher than others.¹⁶ However, the fact that both methods indicated similar concentrations suggested that differences with respect to the other publications might be because of different cultivation conditions, processing, mushroom strains, *etc.* Other mushroom species such as *Marasmius oreades*, belonging to the same family as *L. edodes* (Marasmiaceae), showed 0.7 mg g⁻¹ eritadenine (according to method 2), only half the shiitake levels, suggesting that closely related species might also be able to be used to synthesize significant amounts of this compound. Eritadenine was also found in other mushrooms belonging to the same order as *L. edodes* (Agaricales) such as *Amanita caesarea* (0.6 mg g⁻¹), *Lyophyllum shimeji*, *Agaricus bisporus* and *Pleurotus ostreatus* (approx. 0.2 mg g⁻¹). But other mushrooms not so closely related, such as *Boletus edulis*, *Morchella conica* and *Lactarius deliciosus*, also contained similar eritadenine concentrations (0.2 mg g⁻¹). Therefore, the presence of the compound in higher concentrations might be only attributed to the *Lentinula* genus.

3.2. Production of eritadenine by shiitake fruiting bodies

A more detailed study about eritadenine biosynthesis was carried out by determining its production within different fruiting body tissues and during their development. The results indicated that differences between tissues were more pronounced when using method 2 than 1. Within the cap, eritadenine was present in higher concentrations in the epidermis than in the dermis or gills (Fig. 1b). The role of this compound in the mushroom metabolism has still not been elucidated although some studies suggested its involvement in defense (as an antibiotic nucleoside) like many other derivatives from the secondary metabolism.^{17,18} If this was the case, it seemed adequate to concentrate the compound in the tissue with direct contact with the environment. The stipe also contained the compound but in lower concentration, being in concordance with previous observations made by Saito *et al.* (1975)¹⁶ where lower levels of eritadenine were found in all the stipes compared to the caps of several shiitake strains.

Moreover, when eritadenine biosynthesis was studied during the development of the fruiting bodies, the results indicated that the metabolite was produced in similar concentrations during their complete growth since no differences were found within developmental stages (Table 2). Although mature mushrooms showed slightly higher eritadenine contents (using method 2) differences with earlier stages were not statistically significant. Therefore, since method 2 was an easier procedure and differences between samples were broader than in method 1, method 2 was selected for further eritadenine quantifications.

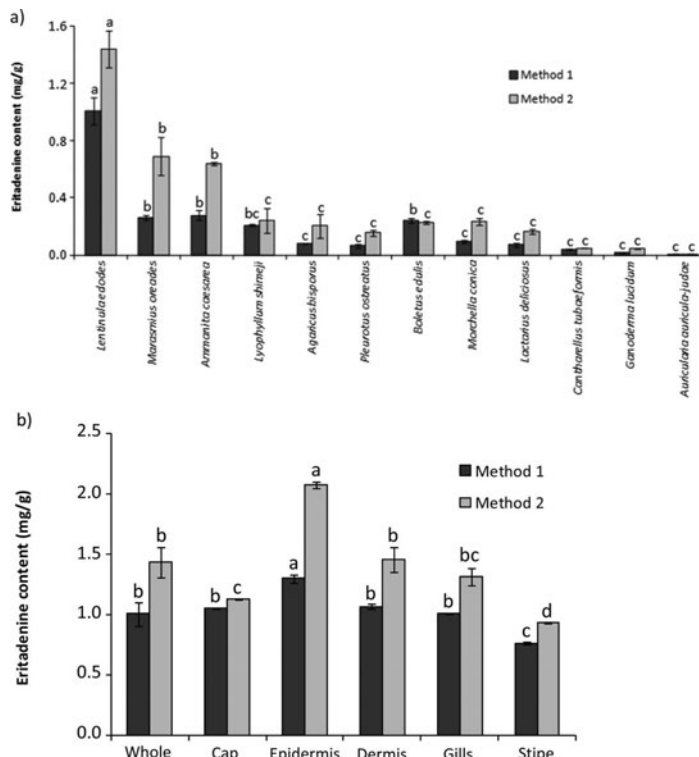


Fig. 1 Eritadenine content (mg per g dry weight) in (a) several mushroom species and in (b) different fruiting body tissues from *Lentinula edodes* determined according to two different methods. Different letters (a–d) denote significant statistical differences between species for the same method ($P \leq 0.05$).

Table 2 Eritadenine content (mg per g dry weight) in different developmental stages of *Lentinula edodes* fruiting bodies. No statistical significant differences were found

Developmental stage	Eritadenine content (mg g ⁻¹)	
	Method 1	Method 2
Immature	1.23 ± 0.01	1.46 ± 0.03
Intermediate	1.01 ± 0.09	1.44 ± 0.13
Mature	1.23 ± 0.01	1.71 ± 0.02

3.3. Effect of traditional culinary treatments on eritadenine stability and bioaccessibility

In order to investigate whether eritadenine might be assimilated by shiitake consumers, the effect of traditional culinary treatments applied to the mushroom before its consumption was evaluated. Furthermore, processed fruiting bodies were subjected to an *in vitro* digestion model mimicking human digestion to determine eritadenine concentrations in the bioaccessible fraction.

The dry heat irradiated during grilling did not significantly affect eritadenine levels compared to raw mushrooms

(Fig. 2a). Apparently, eritadenine was heat stable as also indicated by previous studies.^{12,13} However, when other cooking treatments involving water as a heat transmitter medium were used, such as microwave or boiling, the eritadenine content was reduced. The temperatures in these processes were milder than in grilling; therefore, its losses might be due to lixiviation into the aqueous medium because of its hydrophilic nature. But when a lipid medium was utilized for frying, a decrease in eritadenine content was also noticed. Perhaps the deeper penetration of the oil through the mushroom tissues brought the higher reached temperature (160 °C) to more internal parts of the mushroom entering more into direct contact with the molecule. When dry heat is used, although the irradiated temperature is higher than that in frying (200 °C), only the skin and the first outer layers of tissue receive such irradiation. The inner parts are cooked by their own water content (meaning maximum 100 °C) because of the cooling effect of water evaporation and because biological materials show very low thermic conductivities. The dry heat reaching the skin induced a Maillard crust that might impair the leaching out of constitutive water containing the eritadenine.

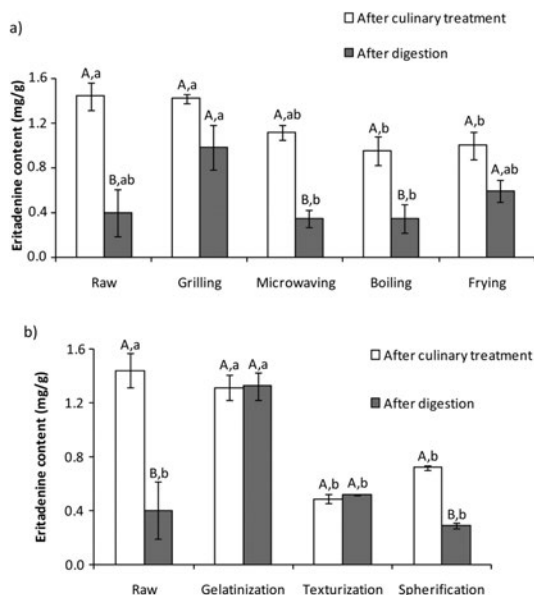


Fig. 2 Eritadenine content (mg per g dry weight) in *Lentinula edodes* subjected to different (a) traditional and (b) modern culinary treatments and after an *in vitro* digestion model. Different letters (a–b) denote significant statistical differences between culinary treatments and (A–B) between the same culinary treatment and after *in vitro* digestion ($P \leq 0.05$).

After the culinary treatments, shiitake mushrooms were treated with digestive enzymes at specific pH values that simulate mastication and stomach and intestinal digestion. The resulting water-soluble digestates were considered as the fraction that might reach the enterocyte layer in the intestine where absorption takes place. The results indicated that, although grilled mushrooms showed similar eritadenine values to raw mushrooms after the digestion, the compound from the grilled mushrooms was more bioaccessible since almost 69.3% of the compound was found in the bioaccessible fraction, while if raw mushrooms were digested only 26.9% was noticed (Fig. 2a). Apparently, the heat treatment partially disintegrated the structural fibers and hyphae facilitating its release into the bioaccessible phase. It could be similar to the effect of the glucanases and chitinases utilized by Enman *et al.* (2007)¹⁴ to extract higher eritadenine concentrations from shiitake tissues.

Moreover, if cooking and digestion are considered, the high temperatures reached during frying were not so detrimental because although part of the eritadenine was lost during the process, it facilitated the release of the remaining levels into the bioaccessible fraction (59.1%) reaching levels higher than the other culinary treatments involving water (30.8% bioaccessibility after microwave cooking and 35.4% after boiling). The further degradation in the latter treatments could be due to the fact that once the eritadenine is leached in the medium

after cooking, it might be more accessible to digestive enzymes. Nevertheless, the eritadenine levels that, in principle, still might reach the enterocytes are similar to those of raw mushrooms.

3.4. Effect of modern culinary treatments on eritadenine stability and bioaccessibility

The effect of novel culinary procedures such as thickening (called 'texturization' by chefs), spherification and gelatinization on the eritadenine concentrations was also tested. The eritadenine content of gelatinized mushroom powder was similar to that of the non-treated powder (raw), suggesting that the mild treatment required to prepare this gel did not negatively influence its levels (Fig. 2b). However, when the mushroom powder was subjected to texturization or spherification, a large reduction of eritadenine was noticed. During texturization high temperatures were used to dissolve the agar-agar but spherification was carried out at room temperature; therefore, degradation by heat could not be the reason for their lower eritadenine content. Moreover, the above results indicated that the compound resisted temperatures higher than 100 °C. Thus, the other possibility might be that, during the culinary procedure, eritadenine could be scavenged by the polysaccharides used to elaborate the dishes (alginate and agar-agar) forming complexes that hindered eritadenine release with the extraction method utilized (method 2). The fact that Enman *et al.* (2007)¹⁴ suggested to use glucanases and chitinases for its extraction might also support this possibility, particularly because if the gel was generated using proteins such as gelatin, an easy release of the compound was noticed.

The eritadenine of the shiitake gels generated using proteins or agar-agar was protected from digestive enzymes as its levels remained the same after digestion (Fig. 2b). However, when spherification was carried out only 40.1% of the spherified eritadenine was bioaccessible; perhaps, the bonds between alginate and eritadenine were stronger than in the other two gels and were resistant to digestion. Nevertheless, its bioaccessible levels were similar to raw mushroom powder with no culinary treatment.

3.5. Biosafety of diets containing eritadenine

The animal intervention was performed with male and female rats administered two different eritadenine doses (high (HE) and low (LE)) and a control group (C) without the metabolite. The LE group received the same eritadenine concentration as previous animal studies since it was indicated as an effective dose with a hypocholesterolemic effect in mice.⁷ The HE group received double the amount to test not only its effectiveness but also its biosafety. However, the compound was administered as a food-grade eritadenine-enriched extract to study its potential as a functional ingredient to formulate novel foods and determine whether the rest of the compounds included in the preparation interfered with or enhanced its bioavailability. During the whole intervention, the appearance, behavior and physiology of animals remained stable. Animal weight gain was recorded during the whole intervention and no differences

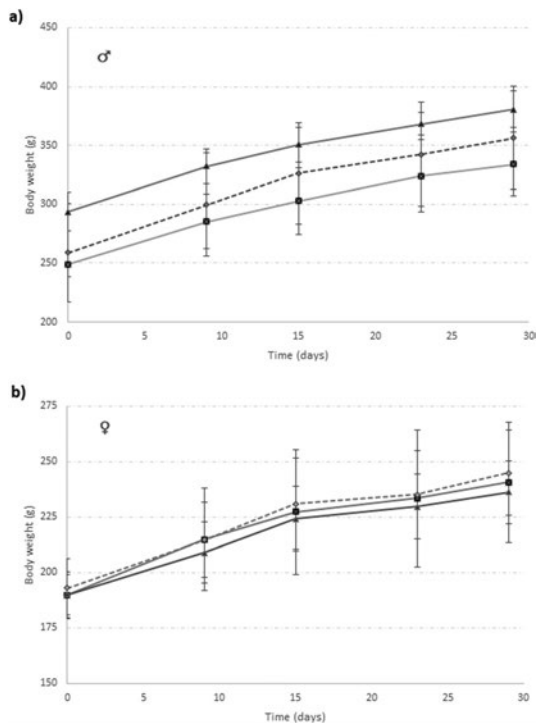


Fig. 3 Body weight gain during eritadenine supplementation. (a) Male and (b) female animals supplemented with high dose (—■—) (0.021 mg per g animal per day) or low dose (—▲—) (0.01 mg per g animal per day) of eritadenine and control group (—◆—). No statistical significant differences were observed between groups.

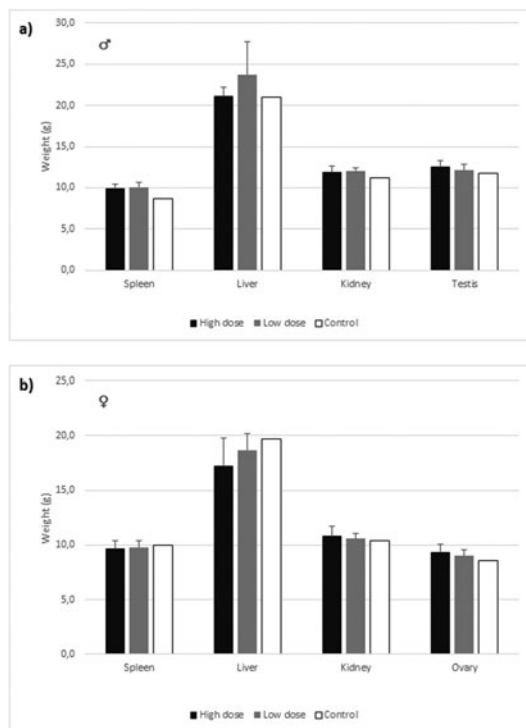


Fig. 4 Organ weight after eritadenine supplementation. Total weight of spleen, liver, kidney and testis/ovary of (a) male and (b) female animals supplemented with a high dose (black bars, 0.021 mg per g animal per day) or a low dose (grey bars, 0.01 mg per g animal per day) of eritadenine and the control group (white bars). No statistical significant differences were observed between supplemented groups.

were observed between groups (Fig. 3). Independently of the studied sex, tissues collected and evaluated did not show damages or differences in weight compared with the control group (Fig. 4). Based on these results, further immunohistochemical analyses were not performed. Plasma samples were determined to evaluate the circulating lipid profile, glucose and biomarkers related to renal and liver function (Table 3). All circulating biomarkers determined were not different from those reported in the control group, except for AST concentrations (aspartate transaminase) that were significantly lower in the LE group than those in the C group (males and females) and total cholesterol that was significantly lower in the LE group than that in the C group but only in males. But a slight increase of uric acid levels was also noticed with increasing extract administration.

3.6 Bioavailability and hypocholesterolemic properties of eritadenine

Eritadenine could be detected in livers using the previously described chromatographic method (Fig. 5). Control rats did not show the eritadenine peak with R.T. 10.5 min while a compound with a spectrum compatible with eritadenine could be

detected in some of the livers treated with both doses. In 3 livers from male rats treated with high eritadenine concentration, the molecule was present within 262.9 to 107.1 μg per g fresh weight while only 2 of those treated with the lower dose showed eritadenine (41.9 and 101.3 $\mu\text{g g}^{-1}$). In the liver from female rats, its concentrations were slightly lower, being detected only in 2 of the livers from rats administered the higher dose (77.4 and 89.6 $\mu\text{g g}^{-1}$) and only 1 of the livers administered the lower dose (67.5 $\mu\text{g g}^{-1}$).

Moreover, an approx. 24% lowering of the atherogenic index TC/HDL was noticed with the lower eritadenine concentration tested compared to the controls (Table 3). This reduction was slightly less pronounced than the approx. 29% noticed in mice for the same eritadenine concentration.⁷ The differences could be due to the different animal/physiological conditions (mice were hypercholesterolemic) or because eritadenine was administered as a standard compound in the previous study. The latter case would indicate that the use of a fungal extract partially impaired the eritadenine bioavailability. Nevertheless, in the same study, *L. edodes* was also directly administered and effective lowering of cholesterol levels was reached (5 and 10%

Table 3 Plasma lipid profile, glucose concentrations and circulating liver and renal damage biomarkers (all values are expressed in mg dL⁻¹ except for ALT and AST that are IU L⁻¹). Statistical significant differences were indicated with the symbol (*)

	Males			Females		
	High dose	Low dose	Control	High dose	Low dose	Control
Total cholesterol (TC)	60.75 ± 2.75	52.75 ± 17.06*	80.00 ± 11.50	71.50 ± 11.73	77.25 ± 9.5	87.00 ± 2.16
HDL-cholesterol	23.25 ± 1.63	18.13 ± 5.45	20.95 ± 2.05	21.77 ± 3.03	23.45 ± 3.14	20.20 ± 1.66
TC/HDL	2.62 ± 0.09	2.89 ± 0.10	3.80 ± 0.25*	3.27 ± 0.12	3.30 ± 0.05	4.33 ± 0.35*
Triglycerides	100.00 ± 33.46	71.50 ± 10.50	109.50 ± 19.02	70.75 ± 28.25	86.50 ± 19.82	103.00 ± 30.74
Glucose	105.50 ± 14.15	85.25 ± 3.30	78.50 ± 16.38	84.25 ± 9.54	101.00 ± 14.49	87.00 ± 9.31
Creatinine	0.29 ± 0.01	0.33 ± 0.06	0.33 ± 0.06	0.31 ± 0.02	0.31 ± 0.03	0.35 ± 0.04
Uric acid	53.75 ± 9.61	41.75 ± 2.22	43.00 ± 5.72	51.25 ± 6.19	47.00 ± 4.55	43.25 ± 3.59
Bilirubin	0.05 ± 0.02	0.07 ± 0.03	0.04 ± 0.03	0.02 ± 0.02	0.05 ± 0.04	0.02 ± 0.01
ALT	38.50 ± 8.19	34.25 ± 2.63	33.00 ± 4.32	43.50 ± 15.8	34.50 ± 9.75	32.75 ± 9.39
AST	98.25 ± 9.74	95.00 ± 12.68*	129.00 ± 14.73	98.50 ± 7.23	86.25 ± 11.15*	119.75 ± 16.88

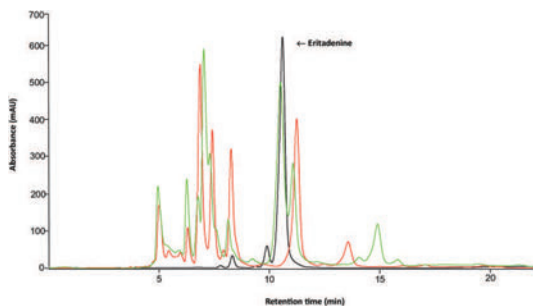


Fig. 5 Chromatograms of liver homogenates obtained from rats supplemented with no eritadenine (red line) or high (green line) eritadenine doses and an eritadenine standard (black line).

L. edodes administration induced, respectively, 10 and 39% TC/HDL reduction). Similar values were obtained in this study for male rats (24 and 31% reduction was noticed for the tested doses, administered as 4.8 and 10% of the diet). Unfortunately, the previous study did not determine the precise eritadenine concentration in the mushroom, nor indicated the animal sex, so no further comparison could be made.

4. Conclusion

Besides *Lentinula edodes*, eritadenine was found in significant amounts in other mushrooms such as *Marasmius oreades* and *Amanita caesarea*. It was synthesized during the complete fruiting body growth, being present in higher concentrations in the skin. The molecule showed certain thermal stability since losses due to common culinary treatments were lower than 35% in all cases. Grilling was recommended more than other methods *e.g.* boiling or microwaving not only to maintain high eritadenine concentrations but because it also enhanced its bioaccessibility, probably by facilitating extraction of the compound from the food matrix or protecting it from digestive enzymes. Moreover, a careful selection of the food additives utilized in molecular gastronomy should be made since the use of hydrocolloids such as

alginates or agar-agar might scavenge the compound, impairing its bioaccessibility. Administration of eritadenine to rats, supplemented as a bioactive ingredient in a normal diet, did not induce damage to any organ or metabolic disorder when added at concentrations up to 21 mg per kg animal per day for 35 days; therefore, it could be considered as safe. It was detected in the liver, suggesting that it was bioavailable and could reach that tissue. The extract reduced the TC/HDL index in serum of male/female rats although it slightly increased uric acid levels. Therefore, functionalization of foods including an eritadenine-enriched extract obtained from shiitake mushrooms to design a hypocholesterolemic product could be possible since apparently it resists culinary processing, it was not toxic, and it was absorbed and was effective at lowering cholesterol in rats. However, clinical trials are still necessary to confirm these effects in humans.

Abbreviations

SAHH	S-Adenosyl-L-homocysteine hydrolase
PC	Phosphatidyl choline
PE	Phosphatidyl ethanolamine
LE	Low eritadenine dose
HE	High eritadenine dose
ALT	Alanine transaminase
AST	Aspartate transaminase
LDL	Low-density lipoprotein
HDL	High-density lipoprotein
TC	Total cholesterol

Conflicts of interest

There are no conflicts to declare.

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Strengths and weaknesses of the aniline-blue method used to test mushroom (1→3)-β-D-glucans obtained by microwave-assisted extractions

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ABSTRACT

The parameters to extract polysaccharide-enriched fractions (PEF) from mushrooms using MAE (microwave-assisted extraction) were adjusted following a full factorial 3² experimental design. The highest yield and total carbohydrate values, using *Lentinula edodes* as model mushroom, were obtained at 180 °C and 30 min. Several mushroom species were submitted to MAE and their PEF yields ranged between 12.1–44.2%. (1→3)-β-D-glucans determination using a conventional fluorimetric method changed depending on the standard utilized. NMR analyses of PEF indicated that the presence of other polysaccharides in the extracts or their specific folding, might impair the proper determination of (1→3) linkages by the fluorophore. Mushrooms from Cantharellales order contained (1→3)-β-D-glucans but they were not detected with the fluorimetric method. Therefore, although the method (after adjustments) was sensitive enough to detect their presence in many mushroom extracts, it cannot be used for all species and it is also not recommended for quantitative determinations.

1. Introduction

Mushroom polysaccharides showed many beneficial properties for human health. They were described as immunomodulatory, anti-bacterial, antidiabetic, anti-inflammatory, hypocholesterolemic agents (Roncero-Ramos & Delgado-Andrade, 2017). Although mushrooms can synthesize a wide variety of polysaccharides, β-D-glucans are those pointed as responsible for most of the biological activities.

β-D-Glucans are present in all mushroom species since they are the major constituents of fungal cell walls contributing to their structure (Ruthes, Smiderle, & Iacomini, 2015). Although linear β-D-glucans were also isolated from certain species, their molecular structures mainly contain a (1→3)-linked backbone chain with substitutions at O-6 by single units of β-D-glucopyranose (Ruthes et al., 2015). Previous studies suggested that the biological properties of these compounds are related to their chemical structure. Thus, characteristics such as the linkage type, polymerization level (Wang et al., 2017) or degree of branching

(Lehtovaara & Gu, 2011) of such molecules might determine not only their biological but also their physico-chemical properties (e.g. solubility) (Lehtovaara & Gu, 2011; Moreno et al., 2016; Thompson, Oyston, & Williamson, 2010). Zhang, Li, Xu, and Zeng (2005) reported that the complexity of the helical conformation (i.e. single or triple) determined the ability of the (1→3), (1→6)-β-D-glucans to inhibit the tumour growth (Meng, Liang, & Luo, 2016). In addition, modified pachyman, a (1→3), (1→6)-β-D-glucan, also showed antitumor properties, after β-(1→6) side chain removal (Wang et al., 2017). These evidences indicated that preserving the structural integrity of fungal β-D-glucans (mostly (1→3), (1→6)-β-D-glucans) during the extraction and analysis processes, might be essential to maintain intact their bioactivities.

Protocols including hot water extractions are the most common methods to obtain fungal polysaccharides (Ruthes et al., 2015; Wang et al., 2017). However, polysaccharides with complex conformations require more aggressive extraction methods such as hot alkali solutions and the use of chloroacetic or concentrated sulphuric acids, but they

Abbreviations: PEF, polysaccharide-enriched fractions; MAE, microwave-assisted extraction; TCH, total carbohydrate; PLE, pressurized-liquid extraction; UAE, ultrasound-assisted extraction; MP, mushroom powder; PSC, polysaccharide; PUL, 13-1,6-β-D-glucan from *P. pulmonarius*

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might also induce undesirable structural modifications (Alzorqi, Singh, Manicham, & Al-Qrimli, 2017; Eskilsson & Bjorklund, 2000; Zhu, Du, & Xu, 2016). Alternatively, new extraction technologies using water such as pressurized liquid extraction (PLE), ultrasound assisted extraction (UAE) or microwave assisted extraction (MAE) were also studied as effective tools for harmless polysaccharide extractions (Smiderle et al., 2017). MAE uses microwaves energy to heat directly the extraction solvent without a heat transfer from the vessels to the solvent. It maintains thermal gradients to minima inducing a more homogeneous temperature through the sample (Eskilsson & Bjorklund, 2000). The high pressure generated during the extraction process maintains water in its liquid state at temperatures higher than its boiling point, accelerating mass transfer of compounds (Li, Dobruchowska, Gerwig, Dijkhuizen, & Kamerling, 2013; Ruthes et al., 2015). Several studies used MAE to extract total polysaccharides from mushrooms (Chen, Shao, Tao, & Wen, 2015; Wang & Li, 2010) however, only a few studied the β -D-glucans content of the obtained extracts (Ruthes et al., 2015; Zheng, Zhang, Gao, & Jia, 2012).

A common method used to study β -D-glucans is based on the siro-fluor ability to bind polysaccharides with (1 \rightarrow 3)- β -D-glucan branches (Alzorqi et al., 2017; Evans, Hoyne, & Stone, 1984; Ko & Lin, 2004). Some reports claimed that such fluorimetric method is selective for (1 \rightarrow 3)- β -D-glucan binding but, it showed certain restrictions that were not always considered, for instance, the polymerization degree, presence/absence of substituents and their tridimensional conformation might modulate the fluorimetric determination (Evans et al., 1984; Ruthes et al., 2015).

Therefore, a three level factorial experimental design was carried out to find the most suitable MAE conditions to obtain β -D-glucans-enriched extracts using *Lentinula edodes* as mushroom model. Afterwards, a screening of other species was performed using similar extraction conditions. The influence of the (1 \rightarrow 3)- β -D-glucan structure on the fluorimetric assay was studied (using also NMR) to remark its limitations and the method was adjusted to improve its accuracy for the detection of β -D-glucans in MAE extracts.

2. Experimental

2.1. Biological material, reagents and standard compounds

Powdered *Lentinula edodes* S. (Berkeley) fruiting bodies with a particle size lower than 0.5 mm and a moisture content less than 5% were purchased from Glucanfeed S.L. (La Rioja, Spain). Dried fruiting bodies, commercially available in local markets, from *Auricularia juda* (Bull. Ex St.Amans) Berck, *Cantharellus cibarius* (Fr.) *Cantharellus tubaeriformis* (Fr.), *Cantharellus lutescens* (Herve.) Fr., *Cantharellus cornucopoides* (L. Ex Fr.) Pers, *Boletus edulis* (Bull. Ex Fr.), *Lactarius*

deliciosus (Fr.), *Pleurotus pulmonarius* (Fr.) Quel, *Pleurotus eryngii* (D.C. Ex Fr.) Quel, *Morchella conica* (Pers.), *Agrocybe aegerita* (Briganti) Singer, *Amanita caesarea* (Scop. Ex Fri.) Pers. Ex Schw. and *Hypsizygus marmoreus* (Peck) H.E. Bigelow were purchased and ground using a Grindomix GM200 Retsch mill (VERDER Group, The Netherlands) as described by Ramirez-Anguiano, Santoyo, Reglero, and Soler-Rivas (2007). Powdered mushrooms (MP) were stored at -20°C under darkness until further use.

Absolute ethanol was purchased from Panreac (Barcelona, Spain) as well as concentrated sulfuric acid. Phenol, sodium borohydride, sodium hydroxide pellets, glycine, aniline blue diammonium salt 95% and hydrochloride acid 37% were obtained from Sigma-Aldrich (Madrid, Spain). Compounds used as standards were two linear β -D-glucans: curdlan (a (1 \rightarrow 3)- β -D-glucan from *Alcaligenes faecalis*, Sigma-Aldrich, Madrid, Spain) and a (1 \rightarrow 6)- β -D-glucan isolated from *A. bisporus* (named B6G) (Smiderle et al., 2013); and two branched (1 \rightarrow 3), (1 \rightarrow 6)- β -D-glucans: schizophyllan from Contipro Biotech (Dolní Dobrouč, Czech Republic) and a chemically characterized glucan (PUL, previously named B1316PP) extracted from *P. pulmonarius* (Smiderle et al., 2008). Moreover, a heteropolysaccharide (mannogalactan, MG) isolated from *P. pulmonarius* (Smiderle et al., 2008) and commonly found in Basidiomycetes, was also used to compare with the β -D-glucans.

2.2. Microwave-assisted extractions (MAE)

Polysaccharides-enriched fractions (PEFs) were obtained using an automated microwave extraction system coupled to a MAS 24 auto-sampler (Monowave 300, Anton Paar GmbH, Graz, Austria). Extractions were performed at 1:30 mushroom powder:water ratio, 850 W power, 2455 MHz frequency and 30 bar pressure as was described by Smiderle et al. (2017). After MAE, the samples were centrifuged and the pellet discarded. An aliquot of the supernatant (200 μL) was used to estimate the total carbohydrate content (TCH) in the MAE extracts. Afterwards, the polysaccharides were precipitated with ethanol under cold conditions following the procedure of Smiderle et al. (2017). Obtained polysaccharide-enriched fractions (PEF) were collected, freeze-dried, weighted and kept at -20°C until further use (Eq. (1), Fig. 1).

$$\text{PEF yield (\%)} = \frac{\text{weight of polysaccharide enriched fraction after MAE precipitation (g)}}{\text{weight of mushroom powder (g)}} \times 100 \quad (1)$$

2.3. Design of experiment (DoE) approach for MAE of *Lentinula edodes*

In order to explore the efficiency of MAE to obtain PEFs, a full factorial three level experimental design (3^k) was selected using *L. edodes* as mushroom model. Two factors (k), extraction temperature

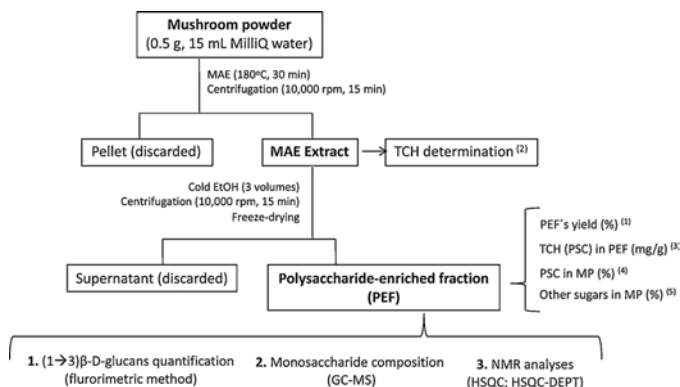


Fig. 1. Workflow of the experiments developed in this work. Superscripts 1–5 correspond to the specific equations used to determine the corresponding values.

Table 1
Full factorial 3² experimental design for *L. edodes* MAE.

Exp. Number	Factors		Response variables	
	Temperature (°C)	Time (min)	TCH in MAE ¹ extracts (mg equiv. Glc/g MP) ²	PEF yield ³ % (w/w)
1	180	5	263.21 ± 18.4	15
2	50	5	107.52 ± 5.8	4.6
3	115	30	88.62 ± 3.12	4.4
4	50	17.5	107.36 ± 8.5	4
5	115	17.5	83.33 ± 2.0	4
6	115	5	98.65 ± 31.0	5
7	115	17.5	86.21 ± 31.4	3.4
8	50	30	116.80 ± 4.9	2.8
9	180	30	290.43 ± 20.6	15.4
10	180	17.5	238.76 ± 12.5	16.4
11	115	17.5	80.38 ± 19.2	3.6

¹ TCH, total carbohydrates content in MAE extracts.

² mg equiv. Glc/g MP, milligrams of equivalent glucose per gram of mushroom powder.

³ PEF yield, polysaccharide yield of the enriched fraction (PEF) obtained from MAE extracts.

and time, were studied and their ranges were set according to the equipment limitations and results reported for other mushroom species (respectively 50–180 °C and 5–30 min) (Smiderle et al., 2017). PEF yield (% g PEF/ 100 g MP) and TCH (mg equivalents of glucose/ g MP) were selected as response variables. Eleven randomized extractions were performed following the parameters indicated in Table 1, i.e. three levels per factor (3²) with two additional central points. Most convenient MAE conditions achieved for *L. edodes* were selected to obtain PEFs from the other mushroom species.

2.4. Determination of carbohydrates from MAE extracts

The total carbohydrate content (TCH) in MAE extracts and PEFs (obtained after precipitation of MAE extracts) (Fig. 1) was measured using the phenol-sulfuric acid method, as detailed by Smiderle et al. (2017). Since only traces of monosaccharides or oligosaccharides might remain in obtained PEFs, in this case, the TCH values indicated the total polysaccharide (PSC) concentration. Calculations were performed as follow (including unit conversions),

$$\text{TCH in MAE extracts (mg/g)} = \frac{\text{equivalents of glucose in MAE extracts (mg)}}{\text{weight of MP (g)}} \quad (2)$$

$$\text{TCH (or total PSC) in PEFs (mg/g)} = \frac{\text{equivalents of glucose in PEF (mg)}}{\text{weight of PEF (g)}} \quad (3)$$

$$\text{PSC in MP after MAE ppt. (\%)} = \frac{\frac{\text{equivalents of glucose in PEF (mg)}}{\text{weight of PEF (g)}} \times \text{weight of PEF (g)}}{10 (\text{unit conversion})} \quad (4)$$

$$\text{Other sugars in MP after MAE ppt. (\%)} = \frac{\text{TCH in MAE extracts}}{10 (\text{unit conversion})} - \text{PSC in MP by MAE precipitation} \quad (5)$$

Supernatants obtained after MAE (Eq. (2), Fig. 1) were diluted for all species as 1:15 (extract: water) except for *L. edodes* (1:5) and for *A. judae* and *P. eryngii* (1:30). TCH in PEFs (Eq. (3), Fig. 1) were diluted for all species as 1:5. Samples were analyzed in triplicate and glucose was used as standard.

2.5. Determination of (1 → 3)-β-D-glucans from PEF

β-D-Glucans were determined by the fluorimetric method firstly described by Evans et al. (1984). This method uses an impurity from the aniline blue stain (sirofluor) as fluorochrome because of its ability to

bind polysaccharides with (1 → 3)-β-linkages (Fig. 1). Thus, the presence of (1 → 3)-β-D-glucans was determined in freeze-dried MAE polysaccharide fractions (PEF) using the method reported by Ko and Lin (2004) with some modifications concerning the sample preparation and the analytical procedure. Briefly, fractions were solubilized in 0.05 M NaOH with 1% NaBH₄ (0.02 mg/mL) to preserve the polysaccharides integrity. Then, samples (300 µL) were mixed with 30 µL of 6 M NaOH and 630 µL of a dye solution (0.1% aniline: 1 M HCl: 1 M glycine/NaOH buffer pH 9.5 33:18:49, v/v/v) and incubated at 50 °C for 30 min in a water bath. Each mixture (250 µL) was transferred to a 96-well plate and analyzed using a M200 Plate Reader (Tecan, Männedorf, Switzerland) with excitation and emission wavelengths of 398 nm and 502 nm respectively. The buffer was freshly prepared before use to avoid degradation.

Fluorescence signals relative to the β-D-glucans content in PEF were obtained at 0.002, 0.01 and 0.02 mg/mL for all previously mentioned species. The standards for β-D-glucans were used in a range of 0–0.02 mg/mL for calibration curves and a solution of 0.05 M NaOH with 1% NaBH₄ was used as blank. Determinations were carried out in triplicate. The selection of the adequate standard for the detection of (1 → 3)-β-D-glucans in a mushroom extract was dependent on recorded fluorescence slopes.

2.6. Analysis of monosaccharide composition by GC–MS

The polysaccharide-enriched fractions (PEF) from MAE extracts (1 mg) were hydrolyzed with 2 M TFA at 100 °C for 8 h, followed by evaporation to dryness (Fig. 1). The dried carbohydrate samples were dissolved in distilled water (100 µL) and 1 mg NaBH₄ was added. The solution was held at room temperature overnight to reduce aldoses into alditols (Sasaki et al., 2008). The product was dried, the excess of NaBH₄ neutralized by the addition of acetic acid and removed with methanol (× 2) under a compressed air stream. Alditols acetylation was performed in pyridine–Ac₂O (200 µL; 1:1, v/v), for 30 min at 100 °C. The pyridine was removed by washing with 5% CuSO₄ solution and the resulting alditol acetates were extracted with CHCl₃. The resulting derivatives were analyzed by GC–MS (Varian CP-3800 gas chromatograph coupled to an Ion-Trap 4000 mass spectrometer), using a VF5 column (30 m × 0.25 mm i.d.) programmed from 100 to 280 °C at 10 °C/min, with He as carrier gas. The obtained monosaccharides were identified by their typical retention time compared to commercially available standards. Results were expressed as mol%, calculated according to Pettolino, Walsh, Fincher, and Bacic (2012).

2.7. Nuclear magnetic resonance spectroscopy

NMR spectra (HSQC and HSQC-DEPT) from PEFs (Fig. 1) was obtained using a 400 MHz Bruker model Avance III spectrometer with a 5 mm inverse probe. The analyses were performed at 70 °C and the samples (30 mg) were dissolved in D₂O (400 µL). Chemical shifts are expressed in ppm (δ) relative to resonance of acetone at δ 30.2 and 2.22 corresponding to ¹³C and ¹H signals, respectively. NMR signals were assigned on the basis of 2D NMR experiment (HSQC) and literature data.

2.8. Statistical analysis

In order to set the experimental conditions and detect the optimal MAE parameters, analysis of variance (two-way ANOVA test) was carried out using the Statgraphics Centurion XVI software (Statpoint Technologies, Warrenton, Virginia, USA). The confidence level was set at 95% (p < 0.05) for all cases.

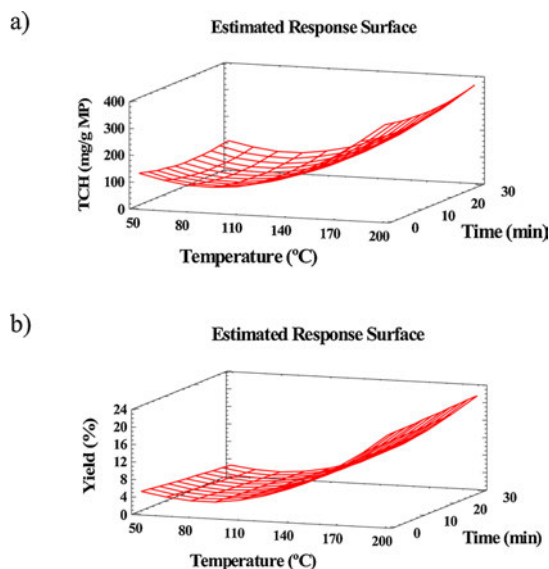


Fig. 2. Estimated response surfaces plots for a) TCH content and b) PEF yields after MAE from *L. edodes*.

3. Results and discussion

3.1. Response surface study of microwave assisted extractions from *L. edodes*

Powdered fruiting bodies from *Lentinula edodes* were submitted to MAE following a full factorial 3^2 experimental design. Two factors, extraction time and temperature, were tested to estimate the more convenient combination to obtain high TCH values and PEF yields (Table 1).

Similar trends in the response surface plots for both variables were obtained (Fig. 2), higher TCH and yields were reached at the highest tested temperatures. The model showed an adequate fitting to experimental data supported by the elevated variability percentage in TCH and yield explained by the model (99.4 and 98.8% respectively). The regression equations fitting to the data were:

$$y_1 = 13.1 - 0.22x_1 - 0.12x_2 + 1.3 \cdot 10^{-3}x_1^2 + 6.7 \cdot 10^{-4}x_1x_2 + 6.0 \cdot 10^{-4}x_2^2$$

$$y_2 = 281.08 - 4.12x_1 - 3.89x_2 + 0.02x_1^2 + 5.5 \cdot 10^{-3}x_1x_2 + 0.1x_2^2$$

where the linear and quadratic effects of both factors (x_1 = temperature and x_2 = time) as well as the interaction between them were included for y_1 (yield) and y_2 (TCH) prediction. Only 'linear' and 'temperature' quadratic terms showed a statistically significant influence on the TCH and yield, following the analysis of the variance results (ANOVA, $p < 0.05$). The factor 'time' as well as the interaction between both factors, were defined as statistically insignificant. However, according to the Pareto charts for each variable response (Supplementary Fig. 1), the term 'quadratic time' affected more to TCH content than to the yield. This effect can be easily visualized in the respective response surface plotting by the curvature along time (Fig. 2a).

The temperature and time values that maximize the TCH and PEF yields of MAEs, within the selected conditions ranges, were 180 °C and 30 min. These values were similar to those previously described for other mushroom species such as *Pleurotus ostreatus* and *Ganoderma lucidum* after a similar extraction procedure (Smiderle et al., 2017). The desirable linear regression obtained by the plot observed vs. predicted values for each variable response validated the model (Supplementary

Fig. 2). Nevertheless, to verify it, three additional microwave-assisted extractions from *L. edodes* were performed at 180 °C and 30 min. Only slightly lower values were observed for TCH (224.4 ± 6.1 mg equiv. Glc/g MP) and higher values for PEF yield ($19.1 \pm 0.3\%$, w/w) were noticed compared to the software predicted values (respectively 278 mg equiv. Glc/g and 15% w/w). Therefore, the predictive model fitted the experimental behavior.

Substantially higher PEF yields were observed after MAE compared to the recoveries previously reported for conventional hot water extractions, i.e. 5.3% were obtained after seven extractions at 100 °C for 200 min (Zhu, Nie, Liang, & Wang, 2013). The higher MAE efficiency could be explained by the more effective disruption of analytes-matrix interactions and an improved mass transference at high temperatures and pressures, resulting in larger recoveries (Plaza & Turner, 2015; Rogalinski, Liu, Albrecht, & Brunner, 2008). However, their higher solubilization might also indicate that MAE could generate some physical changes in the tertiary conformations of the polysaccharides enhancing their extractability (Wang et al., 2017) and/or that some chemical changes such as partial hydrolysis might occur as indicated by other studies (Ookushi, Sakamoto, & Azuma, 2006; Synysya & Novak, 2014). Nevertheless, since the extraction yield steadily increased and no drastic changes were observed/predicted at least up to 180 °C, the latter possibility seemed unlikely.

3.2. Microwave-assisted extractions from other edible mushrooms

MAE were also carried out at 180 °C and during 30 min for the other selected species since with these parameters, the highest TCH and PEF yields were obtained for *L. edodes* and, according to Smiderle et al. (2017), they were also appropriate for other two mushrooms. The yields of obtained PEFs from the selected species ranged between 12.1 (*Cantharellus cibarius*) and 19.1% (*L. edodes*) except for *Auricularia judea* that showed extremely high yields (Table 2). However, the MAE extracts that showed higher TCH values besides *Auricularia judea* were *Pleurotus eryngii*, *Lentinula edodes* and *Boletus edulis* indicating that the latter two species contain higher levels of monosaccharides and oligosaccharides than the rest of species. In the case of *A. judea*, results indicated that almost half of the dry matter from the mushroom powder could be extracted using MAE obtaining PEFs with 96.4% polysaccharides. *L. edodes* yields were in the range of the previously observed values (15.4%) as also noticed for the other species i.e. *Armillaria luteovirens* (8.40–8.34%) (Chen et al., 2015), *G. lucidum* (11.2%) (Smiderle et al., 2017), *Fomitopsis ulmaria* (8.36%) (Zhao, Tang, Liu, & Zhang, 2014) etc. Other mushrooms such as *Morchella conica* showed PEF yields (16.5%) slightly higher than other MAE obtained extracts previously reported (5.86%) (Xu et al., 2018) and *Pleurotus pulmonarius* showed lower values than other related species such as *P. ostreatus* (32.4%) (Smiderle et al., 2017). However, the different extraction conditions such as shorter times and/or different mushroom varieties or cultivation conditions, might be the reason for the noticed differences.

The TCH content determined in PEFs indicated their polysaccharides concentration (since only traces of monosaccharides or oligosaccharides might be present after the precipitation procedure). Results indicated that, indeed, PEFs were fractions with high polysaccharide content except for *Craterellus cornucopioides* (40.3%). PEF obtained from mushrooms such as *Amanita caesarea*, *Agrocybe aegerita* or *Hypsizygus marmoreus* contained 63.6–68.2% PSCs. The rest of the PEF weight might be proteins (perhaps bound to polysaccharides as glucoproteins or proteoglycans) as some of them might also precipitate with the ethanol concentrations utilized.

For certain fungal species, MAE seems to be a more efficient method to obtain polysaccharides than conventional methods or other advanced techniques but not for all. Pressurized water extractions (PWE) extracted higher polysaccharide amounts than MAE from *P. ostreatus* but not significant differences were noticed when the polysaccharides were extracted from *G. lucidum* (Smiderle et al., 2017). Moreover, *L.*

Table 2

Total carbohydrate contents (TCH) in MAE extracts (mg/g), yields of obtained polysaccharide-enriched fraction (PEF) after MAE precipitation, TCH (mg/g) in PEF, PSC (%) in PEF and PSC (%), as well as other sugars, after MAE precipitation.

Species	TCH in MAE extracts (mg/g) [†]	PEF yield % (w/w) [†]	TCH in PEF(mg/g) [*]	PSC in PEFs (%)	PSC in MP after MAE precipitation(%)	Other sugars in MP after MAE precipitation (%)
<i>A. judea</i>	485.5 ± 11.0	44.2 ± 1.3	964.6 ± 65.5	96.4	42.64	5.91
<i>A. aegerita</i>	175 ± 3.9	17.6 ± 0.3	682.7 ± 42.5	68.2	12.02	5.48
<i>A. caesaria</i>	180.6 ± 5.5	13.9 ± 1.2	636.4 ± 37.2	63.3	8.85	9.21
<i>B. edulis</i>	226.9 ± 12.8	12.3 ± 0.1	588.8 ± 8.8	58.8	7.24	15.45
<i>C. cibarius</i>	140.3 ± 31	12.1 ± 0.3	567.5 ± 49.6	56.7	6.87	7.16
<i>C. cornucopioides</i>	87.19 ± 7.9	15.3 ± 0.6	403.4 ± 1.7	40.3	6.17	2.55
<i>C. lutescens</i>	157.4 ± 18.9	15.7 ± 0.1	608.8 ± 40.7	60.8	9.56	6.18
<i>C. tubaeformis</i>	91.3 ± 3.4	16.4 ± 0.1	511.1 ± 34.8	51.1	8.38	0.75
<i>H. marmoreus</i>	184.8 ± 1.0	12.8 ± 1.4	681.5 ± 79.2	68.1	8.72	9.76
<i>L. deliciosus</i>	140.8 ± 11.0	17.8 ± 0.1	561.2 ± 26.5	56.1	9.99	4.09
<i>L. edodes</i>	290.43 ± 20.6	15.4 ± 0.4	563.7 ± 33.6	56.3	8.68	20.32
<i>M. conica</i>	143.4 ± 3.5	16.5 ± 0.9	552.4 ± 53.1	55.2	9.11	5.23
<i>P. eryngii</i>	301.2 ± 16.0	16.8 ± 0.2	593.8 ± 30.1	59.3	9.98	20.14
<i>P. pulmonarius</i>	166.6 ± 3.1	15.4 ± 0.3	585 ± 10.6	58.5	9.01	7.65

* n = 3 independent colorimetric measurements for TCH determination.

† n = 2 independent MAE extractions.

edodes showed a 21% PSCs extraction yield using PWE at higher temperatures (200 °C and 10.7 MPa) but 4.71% was recovered when extraction was carried out at 150 °C (Zhu et al., 2013), values very similar to MAE where a 19.1% yield was noticed. Ultrasound-assisted extractions (UAE) were also tested using several mushrooms however, they were usually less effective than MAE, for instance UAE from the latter mushroom extracted 9.75% PSCs, meaning 1.6 fold increase compared to conventional hot water extraction but still lower than observed using MAE (Zhao, Yang, Liu, Zhao, & Wang, 2018).

3.3. Adjustments of the fluorimetric method for fungal (1→3)-β-D-glucans determination

The fluorimetric method is based on the sirofluor preference for binding to (1→3)-β-D-glucans. Although an increased signal emission intensity can be expected with higher number of these branches, there is not a direct correlation since the fluorescence intensity is affected by the polysaccharide structure (Evans et al., 1984). Ko and Lin (2004) also indicated the influence of the structure and conformation of (1→3)-β-D-glucans to the fluorescence profiles of nine standards, e.g. pachymanan, yeast glucans, curdlan. Therefore, the maintenance of the β-D-glucan native structure during their extraction process is critical for a reliable identification and quantification. However, in previous studies, insufficient attention was given to sample preparation to keep the integrity of these molecules, usually a conventional protocol was followed (Ko & Lin, 2004). Thus, the fluorimetric method used to determine fungal (1→3)-β-D-glucans was adapted at several stages, from the sample preparation to the selection of the adequate standard compound for the β-D-glucan quantification.

The PEFs obtained with MAE were not completely soluble in water at room temperature but in alkalis. However, a careful post-extraction treatment of the samples should be carried out, avoiding drastic changes that could compromise the chemical integrity of the β-D-glucans. Treatment with sodium hydroxide improve β-D-glucans separation from impurities and facilitate their quantification but could modify their helical 3-D conformations (Lehtovaara & Gu, 2011; Young, Dong, & Jacobs, 2000) and in high concentrations could damage even the primary structure. Therefore, a solution of 0.05 M instead 1 M NaOH (Ko & Lin, 2004) was used to dissolve β-D-glucans since the lower concentration was able to dissolve the samples and standards. NaBH₄ (1% w/v) was also added to the NaOH solution to protect the polysaccharide chains from degradation (Whistler & BeMiller, 1958). Indeed, addition of NaBH₄ enhanced 17% the fluorescence intensity of curdlan compared to a similar solution without the reducing agent. Moreover, previous studies remarked the importance of the incubation

step at 80 °C for 30 min to enhance the complex between sirofluor and the polysaccharides with (1→3)-β-D-glucopyranose-linkages (Ko & Lin, 2004). However, if lower temperatures were tested (down to 50 °C), no differences in fluorescence intensity were noticed therefore, fifty degrees were selected to protect the polysaccharides from thermic depolymerization.

Isolated polysaccharides including the β-D-glucans-linkages frequently described in mushrooms were selected as representative standards to adjust the fluorimetric method. When these β-D-glucans were treated according to the modified method, linear (1→3)-β-D-glucans such as curdlan emitted intense fluorescence, but moderate fluorescence was noticed for branched (1→3)-(1→6)-β-D-glucans (Fig. 3a). Schizophyllan and PUL were both (1→3)-β-D-glucans with (1→6)-β-D-Glc unit branching respectively every 3 and 2.7 residues of the backbone chain (Smiderle et al., 2008; Zhang, Kong, Fang, Nishinari, & Phillips, 2013) and therefore, they showed slight differences in their degree of branching (DB) (0.33 for schizophyllan and 0.37 for PUL). Moreover, when β-glucans from *Saccharomyces cerevisiae* were analyzed (DB = 0.03–0.2) (Synytsya & Novak, 2013), an intermediate fluorescence was noticed between curdlan (DB = 0) and schizophyllan suggesting that higher DB might fold the molecules more compact or tighter, impairing the binding of the fluorophore to the (1→3)-linkages of the backbone chain. This effect might result in a fluorescence reduction and indeed, PUL showed a slightly lower fluorescence intensity than schizophyllan. Computer modeling studies using glucans with different configurations (α or β) and linkages, suggested that (1→6)-β-D-glucans might show highly flexible 3D conformation due to an easy rotation freedom between glucose residues, while (1→3)-β-D-glucans showed helical and flexible conformations (including also glucans with O-6-branches) (Zhang, Cui, Cheung, & Wang, 2007). These observations indicated that the helical conformation was essential for the sirofluor fluorescence stimulation. Thus, a linear (1→6)-β-D-glucan (B6G) and a mannogalactan (MG) were also tested under the adjusted conditions and both lacked fluorescence. This result confirmed that still after the protocol modifications, sirofluor was binding to β-D-glucans with helical conformations including linear or/and branched (1→3)-β-D-glucans.

Since each mushroom species synthesize its own particular set of β-D-glucans, a wide variety of primary structures, degrees of branching and conformations are described (Synytsya & Novak, 2013). When the PEF obtained by MAE from several selected species were analyzed to detect the fluorescence of their β-D-glucans, a range of responses was noticed (Fig. 3b), leading to slightly similar slope values. The lowest and highest data were respectively for *M. conica* and *A. caesarea* (Table 3) showing fluorescence values closer to PUL than to

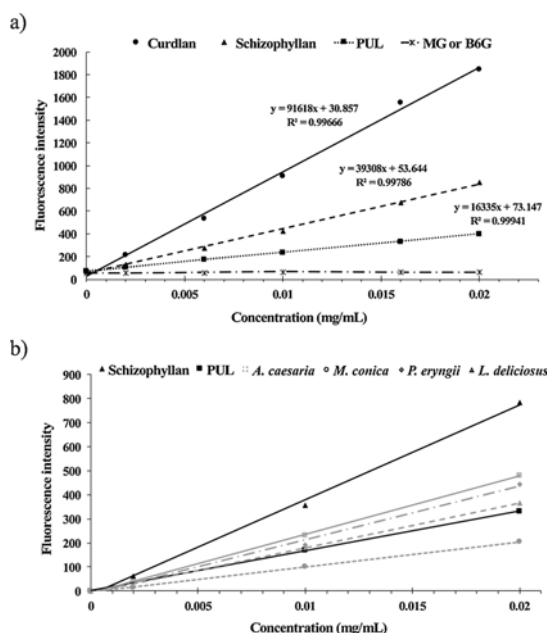


Fig. 3. Fluorescence intensity of a) standard β -D-glucans and a mannogalactan (MG) b) the PEFs extracted by MAE from several mushroom species. The regression equation for the standards is included.

schizophyllan or curdlan. Obviously, the extracts contained many β -D-glucans showing several structures and conformations that were all contributing to the fluorescence intensity while signals from curdlan and schizophyllan are exclusive. However, only curdlan (linear (1 \rightarrow 3)- β -D-glucan) is frequently used as standard for the β -D-glucan quantification regardless the mushroom source and most of the edible species (basidiomycetes) contain larger amounts of branched (1 \rightarrow 3),(1 \rightarrow 6)- β -D-glucans than (1 \rightarrow 3)- β -D-glucan (more common in ascomycetes) (Ruthes et al., 2015). Thus, by using curdlan as standard, the β -D-glucan concentration of many mushroom species might be underestimated or misunderstood. For instance, the β -D-glucan content for *A. aegerita* PEF were 140.1 mg/g using curdlan and 522.3 mg/g using a (1 \rightarrow 3),(1 \rightarrow 6)- β -D-glucan (PUL) as standards (Table 3). For most of the tested species,

results obtained using curdlan or PUL were significantly different (one-way ANOVA analysis ($p < 0.05$) (Table 3). Thus, since the major compounds in basidiomycetes are branched (1 \rightarrow 3),(1 \rightarrow 6)- β -D-glucans, it should be expected a higher contribution of these structures to the total polysaccharide fractions than linear glucans. Therefore, PUL might be a more suitable compound to be used as standard than curdlan, even schizophyllan could also be adequate and more convenient since it is commercially available and would induce a lower quantification error than curdlan (Fig. 3b).

For the same reason, depending on whether PUL or curdlan are used as standard (Table 3), the PEFs composition might be completely different. For instance, the (1 \rightarrow 3)- β -D-glucans represented 14.8% of the polysaccharides from *A. judea* PEF when curdlan is used as standards while using PUL, 63.2% of the PEF polysaccharides were (1 \rightarrow 3)- β -D-glucans. Similarly, the β -glucan percentages found in PEFs seemed to be highly underestimated in other species such as *A. aegerita*, *H. marmoreus*, *M. conica* etc. These results could be supported by those previously reported by Synytsya and Novak (2013) and Smiderle et al. (2011, 2017) where β -D-glucans were found in higher concentrations than other polysaccharides such as α -glucans, chitins, heteropolysaccharides etc. If β -D-glucans are quantified using curdlan their contribution to the total polysaccharide values is quite low to be the predominant compound within the polysaccharide fractions.

However, quantification using PUL could also overestimate their contribution to the polysaccharides content because in the case of species such as *A. caesarea*, *P. eryngii* and *B. edulis* the estimation of β -D-glucans was almost 2-fold the amount of total polysaccharides. Nevertheless, the phenol-sulfuric acid method estimates the PSC concentration mainly because glucose residues are generated with the digestion, if other monosaccharides (xylose, mannose, galactose, etc.) are generated, the method precision decreases. These results bring a suspicion about the suitability of the fluorimetric method for the quantification of β -D-glucans in a complex polysaccharide matrix.

On the other hand, the fluorimetric determinations (independently of the utilized standard) indicated that the polysaccharides detected in PEFs obtained from mushrooms belonging to the Cantharellales order (*Cantharellus lutescens*, *Cantharellus cibarius*, *C. cornucopioides* and *C. tubaeformis*) (Table 2) might be different than (1 \rightarrow 3)- β -D-glucans, or contain also other polysaccharides interfering in the sirofluor complexation, as no fluorescence was detected (Table 3).

3.4. Chemical characterization of the PEFs extracted by MAE

In order to study the real β -D-glucans contribution to the

Table 3

Fluorescence (emission at 503 nm) of PEFs extracted by MAE from several edible mushrooms and their (1 \rightarrow 3)- β -D-glucans concentration depending on the standard utilized (curdlan or PUL). n = 2 MAE extracts per species; n = 2 fluorimetric measurements per PEF; n.d. no signal detected. The linear fitting of the curves (R^2) was 0.99 for all the mushroom species.

Species	Fluorescence results(AU)				(1 \rightarrow 3)- β -D-glucans (mg/g) in PEFs		(1 \rightarrow 3)- β -D-glucans of PSC (%)	
	0.002 mg/mL	0.01 mg/mL	0.02 mg/mL	Curve slope	Curdlan	PUL	Curdlan	PUL
<i>A. judea</i>	30.25 \pm 11.5	159.5 \pm 56.6	295 \pm 86.3	14829	142.7 \pm 49.3	610.0 \pm 234.1	14.8	63.2
<i>A. aegerita</i>	18.7 \pm 6.6	135.7 \pm 11.1	298.2 \pm 15.3	14637	140.15 \pm 8.62 [*]	522.6 \pm 37.0 [*]	20.5	76.5
<i>A. caesarea</i>	37 \pm 7.7	231 \pm 43.3	481.5 \pm 78.0	24266	245.94 \pm 43.1 [*]	1249.9 \pm 241.6 [*]	38.6	196.4
<i>B. edulis</i>	37.7 \pm 10.6	207.5 \pm 55.0	446.2 \pm 110.6	22401	229.21 \pm 63.2	1020.3 \pm 300.1	38.9	173.3
<i>C. cibarius</i>	n.d.	n.d.	n.d.		n.d.	n.d.	–	–
<i>C. cornucopioides</i>	n.d.	n.d.	n.d.		n.d.	n.d.	–	–
<i>C. lutescens</i>	n.d.	n.d.	n.d.		2.12 \pm 2.12	n.d.	0.3	–
<i>C. tubaeformis</i>	n.d.	n.d.	n.d.		n.d.	n.d.	–	–
<i>H. marmoreus</i>	17.2 \pm 4.3	115 \pm 10.0	250.7 \pm 33.0	12643	120.0 \pm 18.5 [*]	543.6 \pm 13.8 [*]	17.6	79.8
<i>L. deliciosus</i>	22.7 \pm 5.3	178 \pm 19.6	368.5 \pm 22.1	18716	184.7 \pm 12.4 [*]	713.7 \pm 53.5 [*]	32.9	127.2
<i>L. edodes</i>	23.5 \pm 12.0	135 \pm 0	311.5 \pm 10.6	15593	152.7 \pm 5.9 [*]	647.1 \pm 28.7 [*]	27.1	114.8
<i>M. conica</i>	15.2 \pm 5.7	102 \pm 23.6	204 \pm 42.3	10325	94.5 \pm 23.1	400.5 \pm 129.5	17.1	72.5
<i>P. eryngii</i>	32 \pm 4.9	192.7 \pm 18.0	445 \pm 37.1	22349	226.4 \pm 20.3 [*]	1140.5 \pm 113.8 [*]	38.1	192.1
<i>P. pulmonarius</i>	18.7 \pm 4.8	156.7 \pm 14.4	337.7 \pm 36.2	17153	167.2 \pm 20.3 [*]	639.4 \pm 87.5 [*]	28.6	109.3

^{*} Data statistically different (one-way ANOVA, $p > 0.05$, 95% confidence level) between standards for the same species.

Table 4
Monosaccharide composition (%) of PEFs obtained by MAE.

Species	Monosaccharides (%) ^a					
	Fucose	Xylose	Methyl-Hexose	Mannose	Galactose	Glucose
<i>A. judea</i>	–	3.4	–	14.3	–	82.3
<i>A. aegerita</i>	2.3	–	–	3.6	5.3	88.8
<i>A. caesarea</i>	1.8	–	–	5.8	5.2	87.2
<i>B. edulis</i>	Tr. ^b	2.3	Tr. ^b	22.8	10.1	64.2
<i>C. cibarius</i>	Tr. ^b	12.7	–	35.9	–	51.2
<i>C. cornucopioides</i>	Tr. ^b	15.8	–	33.5	5.9	44.5
<i>C. lutescens</i>	Tr. ^b	13.7	–	38.0	5.3	42.7
<i>C. tubaeformis</i>	Tr. ^b	21.8	–	29.1	4.7	44.1
<i>H. marmoreus</i>	Tr. ^b	1.8	–	9.9	5.5	82.7
<i>L. deliciosus</i>	Tr. ^b	3.1	–	12.0	6.5	78.2
<i>L. edodes</i>	1.6	2.1	–	14.3	4.9	77.0
<i>M. conica</i>	–	–	–	10.3	4.0	85.7
<i>P. eryngii</i>	–	–	2.5	10.8	4.9	81.8
<i>P. pulmonarius</i>	Tr. ^b	1.3	2.6	8.9	6.8	79.8

^a % of peak area relative to total peak areas, determined by GC–MS.

^b Trace amounts $\leq 0.5\%$.

fluorescence intensity noticed (and to the total polysaccharide values of the PEFs) as well as the method quantification accuracy, the monosaccharide composition and structure of PEFs were monitored.

The PEFs that exhibited fluorescence contained more than 64% glucose in their monosaccharide composition (Table 4). Species that did not show fluorescence, belonging to the genus *Cantharellus*, showed lower glucose content (51.4–42.9%) but considerable amounts of mannose (29.1–38 %) and xylose comparing to the other species. The mushroom from the Boletales order (*B. edulis*) showed higher galactose levels than the rest of selected species. Fucose monosaccharide was only found in few species at levels below 3%. Moreover, methyl-hexose was detected in Pleurotaceae mushrooms (*P. eryngii* and *P. pulmonarius*) and in *B. edulis* in low amounts. Therefore, the slight differences observed in the monosaccharide composition are not consistent enough to explain the different slopes noticed in the fluorescence regression equations. For example, *P. eryngii* and *H. marmoreus* showed similar monosaccharide composition (~82% glucose, ~5% galactose and ~10% mannose) however, the regression slope of the first one was 1.8 fold the

other, suggesting that sugar moieties might not be highly involved in the fluorescence observed (Tables 3 and 4). Wise to take into consideration was the fact that those mushrooms showing high β -glucan contents (even higher than their PSC values) i.e. *A. caesarea* or *P. eryngii* also showed high glucose contents (above 80%). The PSC levels of the Chantarelle family might also be underestimated because they contained large amounts of other monosaccharides that are not glucosides (i.e. *C. lutescens* contains 38% mannose and 13.7% xylose) adding larger errors to the colorimetric determination.

Signals observed in the HSQC spectra from PEFs were in concordance with the monosaccharides determined for each species. The most representative spectra are compiled in Fig. 4, while the others are supplied as supplementary material (Supplementary Fig. 3). With exception of *M. conica*, spectra from *A. caesarea*, *C. lutescens*, *L. edodes* (Fig. 4a–c), *A. aegerita* or *B. edulis* (Supplementary Fig. 3), showed intense signals at δ 102.8/4.52 and 102.8/4.72 relative to C-1 of β -D-Glcp; at δ 85.0/3.75 relative to C-3 O-substituted and; at δ 69.0/4.19 and 69.0/3.86 relative to CH₂ O-substituted of the same units. These data suggest the presence of β -D-glucans (1 \rightarrow 3)-(1 \rightarrow 6)-linked (de Jesus et al., 2017). The O-6 substitution was confirmed by inversion of CH₂ signals of DEPT-HSQC experiment (data not shown). Linear (1 \rightarrow 3)- β -D-glucans signals could be overlapped to the branched β -D-glucans, therefore a separation process might be required to quantify each of them. Besides, signals at δ 99.8/5.36 and δ 77.7/3.64 were intense in *L. edodes* (Fig. 4c) and *M. conica* (Fig. 4d) spectra, characteristic of C-1 of α -D-Glcp and C-4 O-substituted, indicating the presence of glycogen (α -1, 4-1, 6-D-glucan), the energy stock of fungi (Synytsya & Novak, 2013). Small intensity signals of α -D-Galp were observed in all spectra at δ 98.0/4.98 and signals of β -D-Manp were observed mainly in *C. lutescens*, *B. edulis*, and *L. edodes* at δ ~101.6/5.14–102.3/5.11. Species from genus *Cantharellus* showed the higher xylose content, that is not commonly observed in mushrooms and its presence was confirmed by signals at δ ~103.6/4.42 in the spectra of all *Cantharellus* species (Figs. 4b and Supplementary Fig. 3). Mannogalactans, fucmannogalactans and xylomannans were already isolated from other basidiomycetes such as *P. pulmonarius*, *Amanita muscaria*, and *Flammulina velutipes* (Ruthes et al., 2013; Smiderle et al., 2006, 2008), which confirms the findings in this study. Considering that β -D-glucans are able to complex with sir-fluor and *Cantharellus* species did not show fluorescence, it is possible that polysaccharides containing xylose and mannose (present in high

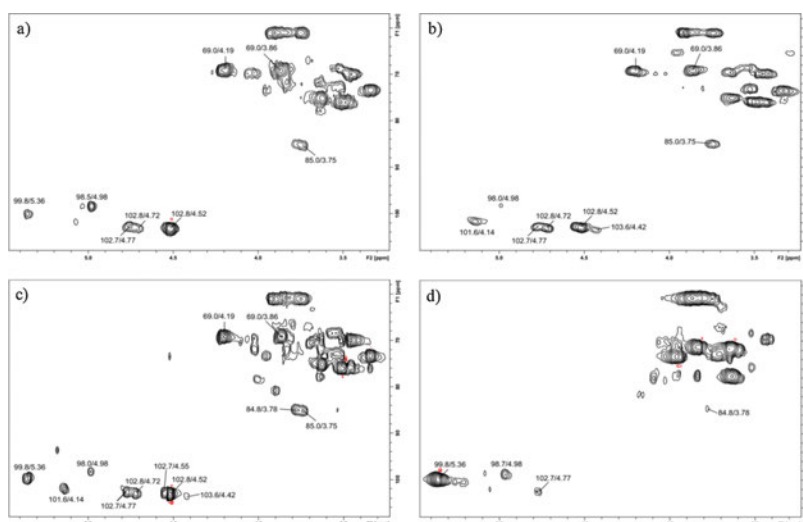


Fig. 4. HSQC NMR spectra of a) *Amanita caesarea*, b) *Cantharellus lutescens*, c) *Lentinula edodes* and d) *Morchella conica*. Experiments were performed in D₂O at 70 °C (chemical shifts are expressed in δ ppm).

amounts in these species) may influence the β -D-glucans 3D conformation present in *Cantharellus* PEFs. These types of polysaccharides were also previously pointed as indirect scavengers of smaller molecules because of their gelling properties so, they could partially attach to sirofluor provoking a lower fluorescence emission (Ngwuluka, Ochekepe, & Aruoma, 2016). Furthermore, *M. conica* showed low fluorescence and its HSQC spectrum presented more intense glycogen signals than signals of β -D-glucans in comparison to the other species, indicating that glycogen might also interfere the proper binding of the fluorochrome to the β -D-glucans structures.

Hence, the fluorimetric method seemed to be sensitive enough for the detection of small amounts of (1 \rightarrow 3)- β -D-glucans in polysaccharide mixtures such a MAE extracts, with limits of detection below 2 μ g/mL. So, it might be used as a fast and easy alternative to determine the presence/absence of (1 \rightarrow 3)- β -D-glucans in complex mixtures, being tentatively applicable to other biological sources. However, to be sure that these linkages are not present in the analyzed samples when lack of fluorescence is observed, more exhaustive qualitative techniques (i.e. NMR) must be performed. No specific correlation was obtained between the fluorescence intensity noticed in the PEFs and their chemical composition suggesting that the fluorescence test should not be recommended for an accurate and trustable quantification of the (1 \rightarrow 3)- β -D-glucans content for any mushroom species. The presence of α -glucans and other polysaccharides, their particular interactions and possible complexing between them or the sirofluor, might under/overestimate their real concentrations depending on the species and standard considered.

4. Concluding remarks

Microwave-assisted extraction is a friendly environmental technology that could be used to obtain polysaccharide-enriched fractions from edible mushrooms. The most suitable extraction conditions seemed to be applicable to many different species obtaining interesting β -D-glucan yields. Although the fluorimetric method resulted appropriate for β -D-glucan detection, this technique must not be used for quantification analysis and a few details should be taken into consideration when carrying it out. For instance, modifications such as reduction of the alkali concentration utilized for experiments, decrease of the incubation temperature and addition of NaBH₄ improved the polysaccharide stability. Utilization of branched (1 \rightarrow 3), (1 \rightarrow 6)- β -D-glucans (particularly PUL or the commercially available schizophyllan) instead of linear (1 \rightarrow 3)- β -D-glucans such as curdlan lowered the experimental error because the fluorescence intensity was influenced by the degree of branching (DB) of the β -D-glucans and most of the mushrooms showed (1 \rightarrow 3), (1 \rightarrow 6)-branched structures in high levels. Moreover, in the case that no fluorescence is observed using the aniline-blue method for mushroom extracts a more exhaustive analysis (i.e. NMR) must be carried out before discarding the sample to avoid misinterpretations (as noticed in the *Cantharellus* genus). Perhaps the presence of other polysaccharides and/or their particular tridimensional configurations negatively affected the preference of fluorophore to bind (1 \rightarrow 3), (1 \rightarrow 6)- β -D-glucans. Deeper studies on the tridimensional folding possibilities of (1 \rightarrow 3), (1 \rightarrow 6)- β -D-glucans and their complexing with other polysaccharides are required to clarify this artefact before using the method for these mushroom species.

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Appendix A. Supplementary data

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Testing the effect of combining innovative extraction technologies on the biological activities of obtained β -glucan-enriched fractions from *Lentinula edodes*

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ABSTRACT

Innovative technologies as ultrasound-assisted extraction (UAE) (550 W, 60% amplitude, 50 °C) or subcritical water extraction (SWE) (200 °C, 11.7 MPa) were more effective than hot water extractions to obtain β -glucan-enriched fractions from shiitake mushrooms. UAE required longer extraction time (60 min) than SWE (15 min). Combination of UAE + SWE or pre-treatment of the raw material with supercritical CO₂ (SFE) (40 °C, 35 MPa, 3 h) before both extractions yielded extracts containing larger β -glucan concentrations. Fluorimetric/colorimetric determinations indicated that obtained fractions contained (1 \rightarrow 3)- and (1 \rightarrow 3), (1 \rightarrow 6)- β -glucans. NMR confirmed their presence as well as (1 \rightarrow 3)- α -glucans and heteropolymers including mannose and galactose. SWE (15 min), SFE + SWE or UAE + SWE extracts showed larger glucose levels and lower mannose and galactose residues than the other extractions suggesting certain extraction specificity towards β -glucans. They also included more chitin-derivatives than UAE. The extracts obtained after combination of technologies partially retained their immunomodulatory properties but they showed high hypocholesterolemic activities according to *in vitro* studies.

1. Introduction

Lentinula edodes is an Asian edible mushroom consumed worldwide and commonly known as shiitake mushroom. Traditionally, it was included in popular remedies to prevent diseases or to promote health and wellbeing and, in the last decades, scientific studies confirmed that indeed, the mushroom contains many bioactive compounds that might reduce the risk of harmful disorders such as cancer, cardio- or cerebrovascular diseases, etc. (Khan, Gani, Khanday, & Massodi, 2018). Fungal β -glucans are compounds thoroughly investigated because they are considered as dietary fiber (together with chitins) with anti-inflammatory, immunomodulatory, antioxidant, anti-tumoral, hypocholesterolemic, antimicrobial activities, etc. (Abreu et al., 2019; Bai et al., 2019; Caz et al., 2016; Friedman, 2019; Khan et al., 2018; Zhang, Li, Wang, Zhang, & Cheung, 2011) and therefore, they might be used to

design novel functional foods.

Mushroom β -glucans are structurally different than those from plants or bacteria since they have a main chain of β -D-glucose units (1 \rightarrow 3)-linked with different branching usually at O-6 position by β -D-glucose units or other oligosaccharides. Their branching degree determines the tertiary structure of the glucan: (1 \rightarrow 3)- β -glucans with few or no branches mainly present a single linear structure while highly branched (1 \rightarrow 3), (1 \rightarrow 6)- β -glucans show a triple helix conformation (Nitschke et al., 2011). In shiitake mushrooms, lentinan is the major (1 \rightarrow 3), (1 \rightarrow 6)- β -glucan and showed many beneficial properties for human health (Kupfahl, Geginat, & Hof, 2006; Suzuki, Takatsuki, Maeda, Hamuro, & Chihara, 1994). Since the integrity of the molecules is important to keep many of their beneficial properties, the extraction methods utilized to generate β -glucan-enriched extracts should be carefully selected because they might modify their molecular weight or

Abbreviations: HWE, hot water extraction; SPE, steam pressurized extraction; UAE, ultrasound-assisted extraction; SWE, subcritical water extraction; SFE, supercritical fluid extraction; MAE, microwave-assisted extraction; HPSEC, high-performance size-exclusion chromatography system; HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase

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tridimensional structure (Benito-Roman, Martín-Cortes, Cocero, & Alonso, 2016).

Although some fungal polysaccharides are polar and easily extractable with water, many β -glucans require aggressive treatments such as hot water or alkaline solutions and others remain in the insoluble residue (Morales, Smiderle, Piris, Soler-Rivas, & Prodanov, 2019; Ruthes, Smiderle, & Iacomini, 2015). Moreover, chitins are water insoluble polymers and only low molecular weight derivatives or degradation products could be extracted using conventional hot water procedures (Morales et al., 2019; Morales, Piris, Ruiz-Rodríguez, Prodanov, & Soler-Rivas, 2018; Ruthes et al., 2015). To obtain chitinous materials, more drastic treatments are required to induce dissolving of other components in alkaline or acid solutions and the precipitation of chitin-enriched fractions (Wu, Zivanovic, Draughon, & Sams, 2004; Wu, Zivanovic, Draughon, Conway, & Sams, 2005). Novel advanced technologies such as ultrasound-assisted extractions (UAE) or subcritical water extraction (SWE) facilitate the extraction of these and other polysaccharides using water as non-pollutant solvent (Rosello-Soto et al., 2016; Marin et al., 2018). Ultrasound-assisted extractions generates ultrasonic waves provoking implosions of the generated cavitation bubbles, causing disruption of fungal cell walls. This treatment enhanced mass transfer and extraction yields (Chemat et al., 2016; Morales, Ruiz-Rodríguez, & Soler-Rivas, 2018; Rosello-Soto et al., 2015) and it was successfully used to extract polysaccharides from several mushrooms (*Ganoderma lucidum*, *Agaricus bisporus*, *Boletus edulis*, etc.) and their by-products (Aguilo-Aguayo, Walton, Vinas, & Tiwari, 2017; Alzorqi, Sudheer, Lu, & Manickam, 2017; Morales, Ruiz-Rodríguez et al., 2018). Pressurized liquid extractions constitute other interesting approach to enhance polysaccharide extraction, particularly SWE. When subcritical pressures are used, the solvent is heated above its boiling point keeping its liquid state and conferring it different properties than those showed at room temperature and atmospheric pressure. SWE modify dielectric constant of water and decrease its viscosity being able to solubilize non-polar compounds such as large size polysaccharides (when temperature is above 100 °C) (Li, Dobruchowska, Gerwig, Dijkhuizen, & Kamerling, 2013; Morales, Ruiz-Rodríguez et al., 2018; Plaza & Turner, 2015; Smiderle et al., 2017). Mushrooms polysaccharide-enriched extracts were obtained by SWE from many species such as *Agaricus bisporus*, *Pleurotus ostreatus*, *Ganoderma lucidum*, etc. (Kodama et al., 2015; Palanisamy et al., 2014; Smiderle et al., 2017).

Besides the previously mentioned, other green technologies were used to extract interesting compounds from edible mushrooms. Supercritical fluid extractions (SFE), particularly those using supercritical CO₂ as solvent, were mainly applied to selectively extract lipids from food matrices because of the low polarity of the solvent (Herrero, Cifuentes, & Ibáñez, 2006). Therefore, it might also be used to remove fat components from fungal extracts enhancing, in a subsequent step, polysaccharide extraction, as described in previous works for *Antrodia camphorata* and *Agaricus blazei* mycelia (Chen et al., 2007; Ker et al., 2005). Combinations of different extraction technologies to obtain β -glucan-enriched extracts were also tested and higher yields than single extractions were usually noticed. For instance, in a complex extraction reactor, the use of pressurized hot water combined with sub/supercritical CO₂ increased 1.26 folds β -glucan yields obtained from *Ganoderma lucidum* than using only pressurized hot water (Benito-Roman, Alonso, Cocero, & Goto, 2016). Moreover, ultrasonic/microwave assisted extractions (UMAE) were also more effective than hot water extractions to obtain bioactive polysaccharides from *Inonotus obliquus* (Chen, Huang, Li, Wang, & Tang, 2010) and combined with enzymes facilitated the extraction even more (Yin, Fan, Fan, Shi, & Gao, 2018).

Therefore, in this work, UAE and SWE were firstly carried out at different extraction times to define the most adequate to obtain β -glucan-enriched extracts from shiitake mushrooms. The extraction yields were compared with more conventional extractions. Afterwards, combinations of UAE and SWE as well as pre-treatments with SFE were

investigated as innovative protocols to improve β -glucan extraction yields. The nature of bioactive polysaccharides from the extracts was determined by different methodologies (enzymatic and colorimetric methods, HPSEC, GC-MS, NMR) and their biological activities studied *in vitro* (hypocholesterolemic and immunomodulatory activities) to investigate whether the extraction procedure modify their beneficial properties.

2. Materials and methods

2.1. Biological material

Powdered *Lentinula edodes* S. (Berkeley) fruiting bodies (particle size < 0.5 mm, moisture < 5%) were purchased from Glucanfeed S.L. (La Rioja, Spain) and stored in darkness at -20 °C until further use.

2.2. Reagents

Absolute ethanol and sulfuric acid (H₂SO₄) were obtained from Panreac and phenol, sodium borohydride (NaBH₄), sodium hydroxide pellets, glycine, D-glucose, glucosamine hydrochloride, aniline blue diammonium salt 95%, hydrochloric acid 37%, acetylacetone, p-dimethylaminobenzaldehyde, trifluoroacetic acid, pyridine, acetic anhydride, copper(II) sulfate (CuSO₄), deuterated dimethylsulfoxide (Me₂SO-d₆), Congo Red, citric acid, RPMI 1640 medium and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich Química (Madrid, Spain). CO₂ was supplied by Air-Liquid, S.A. (Madrid, Spain), schizophyllan was obtained from Contipro Biotech (Dolní Dobruška, Czech Republic) and synthetic soluble starch was acquired from Scharlab (Barcelona, Spain).

2.3. Supercritical CO₂ extraction pre-treatment (SFE)

Shiitake powder (253 g) was mixed with 1.9 kg of 6 mm diameter stainless steel spheres (ratio 1:1 (v/v) powder:spheres) in a 2 L extraction cell connected to a supercritical fluid extraction plant (model SF2000, TharTechnology, Pittsburgh, PA). Pressurized CO₂ was applied into the loaded cell at 35 MPa and 40 °C with a 3.6 kg/h recirculating flow during a total extraction time of 3 h as they were the conditions described as adequate for fungal lipids (Morales et al., 2017; Morales, Piris et al., 2018). The lipophilic material solubilized in supercritical CO₂ was removed in separators (García-Risco, Vicente, Reglero, & Fornari, 2011). After those 3 h, the pressurized material remaining in the extraction cell (called SFE fraction) was separated from steel spheres by sieving in a sieve shaker (Cisa BA200 N, Barcelona, Spain) and stored at -20 °C until further use.

2.4. Ultrasound-assisted extractions (UAE)

Shiitake powder (1 g) was mixed in MilliQ water (100 mL) and submitted to sonication (Branson SFX550 Digital Sonifier 550 W, Branson Ultrasonics, USA) using an ultrasonic probe (1/2") and selecting 60% amplitude for sonication output. During processing, the temperature was maintained at 50 °C using a water bath with ice and a thermometer and samples were taken after 15, 30, 45 and 60 min extraction in duplicate. The obtained mixtures were submitted to vacuum filtration (through Whatman filter paper no. 1) to separate the soluble fraction from the insoluble residue. Afterwards, soluble fractions were lyophilized (UAE fractions) using a freeze-dryer LyoBeta 15 (Telstar, Madrid, Spain) and stored at -20 °C until they were further processed to obtain polysaccharide-enriched extracts or submitted to subcritical water extractions.

The UAE fractions were mixed with water (50 g/L) and three volumes of ethanol to induce polysaccharide precipitation and left incubating overnight at 4 °C. The precipitates were collected by centrifugation (10,000 rpm, 10 min, 4 °C) in a Thermo Scientific Heraeus

Multifuge (Thermo Fisher Scientific, Madrid, Spain) and freeze-dried. The obtained polysaccharide-enriched extracts (UAE extracts) were stored at -20°C until further analysis.

2.5. Subcritical water extractions (SWE)

Shiitake powder (0.5 g) was mixed with washed sea sand (Panreac, Barcelona, Spain) at ratio 1:8 (mushroom:sand, w/w) and placed in an extraction cell (11 mL) covered with cellulose filters (Dionex Corporation, USA) from an Accelerated Solvent Extractor (ASE) 350 (Dionex Corporation, USA). Extractions were carried out in duplicate with MilliQ water at 200°C and 11.7 MPa but using different extraction times (15, 30, 45 and 60 min). Temperature was fixed at 200°C as it was previously pointed as the ideal temperature to extract polysaccharides from shiitake mushrooms (Palanisamy et al., 2014). The obtained mixtures were vacuum filtrated and the soluble fractions were freeze-dried (SWE fractions) and used to generate polysaccharide-enriched extracts (SWE extracts) as above described.

2.6. Conventional extractions

Two different conventional extraction methods were followed to compare with the previously mentioned advanced technologies. One was a simple hot water extraction (HWE) and the other was a method frequently used to obtain polysaccharides using an autoclave (steam pressurized extraction, SPE).

Shiitake powder (0.5 g) was mixed in MilliQ water (50 mL), placed into a water bath at 100°C and vigorously stirred during 15, 30, 45 and 60 min. Incubations were carried out in duplicate. Afterwards, the obtained mixtures were vacuum filtrated and the soluble fractions freeze-dried. Obtained HWE fractions were later submitted to polysaccharide extraction as previously described to obtain hot water extracts (HWE extracts).

Similarly, the same shiitake mixture was heated at 120°C for 20 min in an autoclave and cooled down to 4°C following the method reported by Jeurink, Noguera, Savelkoul, and Wichers (2008). After the steam pressurized extraction (SPE), the mixture was also submitted to vacuum filtration and the soluble fraction freeze-dried (SPE fraction) and further processed to obtain SPE extracts as above indicated for HWE extracts.

2.7. Combined extractions

Sequential extractions were also tested as indicated in Fig. 1 by combining SFE pre-treatment (35 MPa, 40°C , 3 h) with ultrasound assisted extraction or subcritical water extraction. Thus, obtained SFE fraction was submitted to UAE (550 W, 50°C , 60% amplitude) during 60 min as previously indicated for shiitake powder to generate a new SFE + UAE fraction that was further processed to obtain a polysaccharide-enriched extract (SFE + UAE extract) as also described for UAE extracts. Similarly, SFE fraction was submitted to SWE (200°C , 11.7 MPa, 15 min) obtaining a SFE + SWE fraction and a SFE + SWE polysaccharide-enriched extract (SFE + SWE extract). Moreover, the UAE fractions (550 W, 50°C , 60% amplitude) obtained after 60 min sonication were also submitted to subcritical water extraction (200°C , 11.7 MPa) during 15 min to obtain the UAE + SWE fractions. Afterwards, polysaccharide-enriched extracts (UAE + SWE extracts) were also generated following the same procedure as previously described for UAE extracts. All these combined extracts were prepared in duplicate and stored as indicated for the simple extracts.

2.8. Determination of carbohydrates

Total carbohydrate content of shiitake, the extracted fractions and the obtained polysaccharide-enriched extracts was determined by the phenol-sulfuric acid method as described in Fox and Robyt (1991).

Total β -glucan concentration was determined in the polysaccharide-enriched extracts using a mushroom and yeast specific β -glucan determination kit (β -glucan Assay Kit Megazyme®, Megazyme, Wicklow, Ireland) following the instructions of the user's manual. Samples absorbance was measured using a Genesys 10 UV spectrophotometer (Thermo Fisher Scientific, Madrid, Spain).

The amount of (1 \rightarrow 3)- β -glucans in the extracts was determined by the fluorimetric method described by Ko and Lin (2004) but including the modifications of Gil-Ramirez, Smiderle, Morales, Iacomini, and Soler-Rivas (2019) and Smiderle et al. (2017). The measurements were performed in a M200 Plate Reader (Tecan, Männedorf, Switzerland) with excitation and emission wavelengths set at 398 and 502 nm respectively. The (1 \rightarrow 3), (1 \rightarrow 6)- β -glucan content of the extracts was analyzed following the colorimetric method (523 nm) described by Nitschke et al. (2011). Schizophyllan was used as standard for both determinations.

Chitin content was determined according to Smiderle et al. (2017). Briefly, precipitated extracts were hydrolyzed with 6 M HCl at 100°C for 2 h. After the hydrolysis, samples were cooled down and adjusted to pH 10.0. Then, hydrolyzed samples (250 μL) were treated as described by Rementeria et al. (1991) and absorbance was measured at 530 nm. A glucosamine hydrochloride standard curve was used for quantification.

2.9. GC-MS analyses

The monosaccharide composition of the polysaccharide-enriched extracts was determined by hydrolyzing the extracts (1 mg) with 2 M trifluoroacetic acid at 100°C for 8 h followed by evaporation to dryness. The dried samples were dissolved in distilled water (100 μL) and NaBH_4 (1 mg) was added. Then, solution was kept at room temperature overnight to reduce aldose into alditols (Sasaki et al., 2008) and later, the samples were dried and the NaBH_4 excess was neutralized by adding acetic acid and then removed with methanol (twice) under a compressed air stream. Alditols acetylation was performed in pyridine-acetic anhydride (200 μL ; 1:1 v/v) for 30 min at 100°C . Pyridine was removed by washing with 5% CuSO_4 solution and the resulting alditol acetates were extracted with chloroform. The samples were injected into an SH-Rtx-5 ms (30 m \times 0.25 mm ID \times 0.25 μm thickness phase). The column was connected to a GC-2010 Plus gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a Combipal autosampler (AOC 5000) and coupled to a triple quadrupole mass spectrometer TQ 8040. The injector and ion source were held at 250°C and helium at 1 mL/min was used as carrier gas. The oven temperature was programmed from 100 to 280°C at $10^{\circ}\text{C}/\text{min}$ with a total analysis time of 30 min. The samples were prepared in hexane with 1 μL being injected with a split ratio of 1:10. The mass spectrometer was operated in the full-scan mode over a mass range of m/z 50–500 before selective ion monitoring mode, both with electron ionization at 70 eV. Selective ion monitoring mode was used for quantification and GCMS solution software (Tokyo, Japan) was used for data analysis. The obtained monosaccharides were identified by their typical retention time compared to commercial available standards. Results were expressed as mol%, calculated according to Pettolino, Walsh, Fincher, and Bacic (2012).

2.10. HPSEC analyses

Polysaccharide-enriched extracts were injected into a high-performance size-exclusion chromatography system (HPSEC) (Waters, Massachusetts, USA) coupled to refractive index detector (Waters). Four gel-permeation Ultrahydrogel columns in series with exclusion sizes of 7×10^6 , 4×10^5 , 8×10^4 and 5×10^3 Da were used. The eluent was 0.1 aqueous NaNO_2 containing 200 ppm aqueous NaN_3 at 0.6 mL/min. The extracts were dissolved in the eluent (1 mg/mL), filtered through a 0.22 μm membrane and then, injected (100 μL loop). Data were analyzed using Astra software version 4.70.

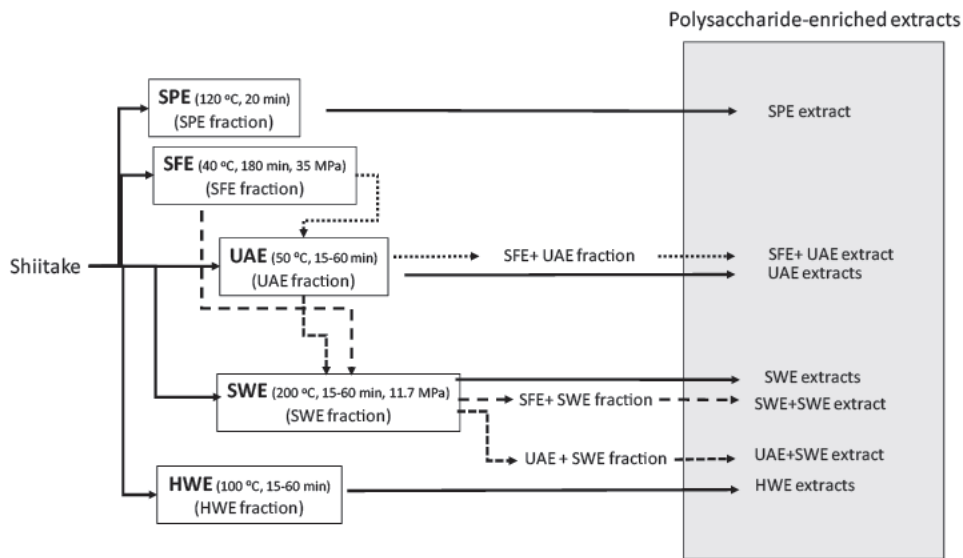


Fig. 1. Extraction workflow followed to obtain different fractions and extracts from shiitake powder. Continuous line indicate single extractions and dotted lines indicate the order followed when two technologies were combined. (SPE, steam pressurized extraction; SFE, supercritical fluid extraction; UAE, ultrasound assisted-extraction; SWE, subcritical water extraction).

2.11. NMR analyses

NMR spectra (^1H , ^{13}C and HSQC-DEPT) from precipitated extracts were obtained using a 400 MHz Bruker model Avance III spectrometer with a 5 mm inverse probe. The analyses were performed at 70 °C and the samples (30 mg) were dissolved in $\text{Me}_2\text{SO}-d_6$. Chemical shifts are expressed in ppm (δ) relative to $\text{Me}_2\text{SO}-d_6$ at 39.7 (^{13}C) and 2.40 (^1H).

2.12. Determination of HMGR inhibitory activity

The polysaccharide-enriched extracts were solubilized in water (50 mg/mL) and applied (20 μL) into a 96-wells plate. Their inhibitory activity was measured using the commercial HMGR (3-hydroxy-3-methylglutaryl coenzyme A reductase) activity assay (Sigma-Aldrich, Madrid, Spain) according to the manufacturer's instructions by monitoring their absorbance change (340 nm) at 37 °C using a 96-wells microplate reader BioTek Sinergy HT (BioTek, Winooski, USA). Pravastatin was used as a control for positive inhibition.

2.13. Macrophage cultures and immunomodulatory testing

The human monocyte THP-1 cell line was obtained from ATCC and cultured with supplemented RPMI 1640 medium (10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin, 2 mM L-glutamine and 0.05 mM β -mercaptoethanol). For differentiation into macrophages, THP-1 cells were seeded (5×10^5 cells/mL) in 24 well-plate with 100 ng/mL phorbol 12-myristate 13-acetate (PMA) and maintained for 48 h at 37 °C under 5% CO_2 in a humidified incubator.

Firstly, the extracts cytotoxicity was evaluated in differentiated macrophages using 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide (MTT) according to Mosmann (1983). Afterwards, the macrophages were washed with PBS and then replaced with serum-free medium containing LPS (0.05 $\mu\text{g}/\text{mL}$) and subtoxic concentrations of the shiitake extracts. After 10 h of incubation, cells supernatants were collected and store at -20 °C until use.

Pro-inflammatory cytokines TNF α (Tumour necrosis factor alpha), IL-1 β (Interleukin 1 beta) and IL-6 (Interleukin 6) were measured in the

supernatants by BD Biosciences Human ELISA set (Aalst, Belgium) following the manufacturer's instructions. The quantification was calculated considering positive controls (cells stimulated with LPS) as a 100% cytokine secretion. The colour generated was determined by measuring the OD at 450 nm using a multiscanner autoreader (Sunrise, Tecan). Experiments were carried out in triplicate.

2.14. Statistical analyses

Differences were evaluated at 95% confidence level ($P \leq 0.05$) using a one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison test. Statistical analysis was performed using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA).

3. Results and discussion

3.1. UAE and SWE as individual extraction methodologies

Polysaccharide extractions using ultrasounds or subcritical water were carried out at different extraction times and compared with conventional methodologies such as hot water extractions (HWE). When the obtained yields were evaluated (Table 1), results indicated that SWEs extracted more material from shiitake powder than UAEs, almost 2 fold more than HWEs at any extraction time and also more than a commonly utilized method to isolate polysaccharides using water at 120 °C for 20 min (SPE) (Jeurink et al., 2008). These results might suggest that the high temperature of the pressurized water (that might have easily penetrated in the hyphae because it still maintains its liquid status) was more effective to dissolve and extract fungal compounds than other methods using lower temperatures such as 120, 100 or 50 °C. Similarly, the propagation of ultrasonic waves might have also provided a greater water penetration into the fungal hyphae than more conventional methodologies using higher temperatures.

HWE yields were higher than reported in previous studies where extractions were carried out for 60 min at 98 °C (Morales, Piris et al., 2018; Morales et al., 2019). However, the value obtained using the SPE method was in the range of previous works (Jeurink et al., 2008).

Table 1

Extraction yields, polysaccharides and other carbohydrates contents obtained in the fractions extracted from shiitake using individual and combined extraction technologies. Different letters (a–g) denote significant differences ($P < 0.05$).

Technology	Extraction time (min)	Yield of extracted matter/100 g shiitake (% w/w)	Yield of precipitated matter/100 g extracted fraction (% w/w)	Yield of precipitated matter/100 g shiitake (% w/w)	Total polysaccharides in extracted fractions (g/100 g)	Other carbohydrates in extracted fractions (g/100 g) [*]
Hot water extraction (HWE)	15	37.99 \pm 5.35 ^{de}	20.29 \pm 2.88 ^e	7.71 \pm 1.09 ^g	5.02 \pm 0.71 ^c	9.63
	30	34.58 \pm 2.22 ^e	19.76 \pm 1.20 ^e	6.83 \pm 0.41 ^g	4.56 \pm 0.27 ^c	18.49
	45	39.96 \pm 0.06 ^{de}	21.18 \pm 0.30 ^{de}	8.46 \pm 0.12 ^g	4.02 \pm 0.06 ^c	30.09
	60	31.24 \pm 0.90 ^e	16.59 \pm 0.52 ^{ef}	5.18 \pm 0.16 ^g	4.78 \pm 0.15 ^c	41.79
Ultrasound-assisted extraction (UAE)	15	45.74 \pm 1.42 ^d	27.26 \pm 0.60 ^d	12.47 \pm 0.27 ^{fg}	8.59 \pm 1.41 ^{bc}	13.74
	30	56.19 \pm 2.81 ^c	30.90 \pm 0.76 ^{cd}	17.36 \pm 0.43 ^e	9.37 \pm 2.92 ^{bc}	21.04
	45	58.52 \pm 2.22 ^c	30.92 \pm 1.95 ^{cd}	18.09 \pm 1.14 ^e	7.76 \pm 1.31 ^c	29.73
	60	60.36 \pm 1.48 ^c	34.56 \pm 3.17 ^c	20.86 \pm 1.91 ^e	10.14 \pm 2.46 ^{bc}	36.50
Subcritical water extraction (SWE)	15	73.21 \pm 0.91 ^{ab}	52.88 \pm 2.12 ^b	38.71 \pm 1.55 ^c	15.54 \pm 2.18 ^{ab}	12.85
	30	75.31 \pm 0.91 ^{ab}	23.16 \pm 0.56 ^{de}	17.44 \pm 0.42 ^e	6.80 \pm 0.94 ^c	31.64
	45	70.58 \pm 3.82 ^b	26.96 \pm 0.99 ^{de}	19.03 \pm 0.70 ^e	7.93 \pm 1.07 ^c	32.51
	60	64.49 \pm 2.62 ^{bc}	20.05 \pm 1.32 ^e	12.93 \pm 0.85 ^f	5.92 \pm 0.67 ^c	27.79
Steam pressurized extraction (SPE)	20	37.96 \pm 0.16 ^{de}	11.42 \pm 0.01 ^f	4.34 \pm 0.01 ^g	5.13 \pm 0.01 ^c	10.16
UAE + SWE	60 + 15	81.20 \pm 1.06 ^a	68.88 \pm 2.33 ^a	55.93 \pm 1.89 ^a	21.14 \pm 1.96 ^a	37.97
SFE + UAE	180 + 60	59.73 \pm 0.44 ^c	49.42 \pm 2.66 ^b	29.52 \pm 1.59 ^d	14.46 \pm 1.97 ^b	34.46
SFE + SWE	180 + 15	74.95 \pm 1.26 ^{ab}	63.54 \pm 1.75 ^a	47.62 \pm 1.31 ^b	19.88 \pm 1.23 ^{ab}	39.27

* Calculated by subtracting total polysaccharide levels to the total carbohydrate values from the extracted fractions obtained before polysaccharide precipitation.

Worth to notice was the decrease observed on SWEs carried out for more than 45 min. It might indicate clogging of the cellulose filter due to the large amount of extracted material. Therefore, if long extraction periods are required, extraction cycles are encouraged since similar studies but using extraction cycles (200 °C, 5 cycles of 5 min) yielded slightly higher values (Palanisamy et al., 2014).

The polysaccharides amount detected in UAE obtained fractions (Table 1) were in concordance with previous works where 9.75% polysaccharides were recorded from *L. edodes* after 21 min extraction (Zhao, Yang, Liu, Zhao, & Wang, 2018). Similarly, the SWE fractions obtained after 15 min extraction showed a slightly higher polysaccharide content than noticed by Zhang et al. (2019) but because only lower temperatures were tested (11.35–12.09%).

After extraction, the obtained fractions were submitted to precipitation to generate polysaccharide-enriched extracts (Fig. 1). Results indicated that the HWE fractions contained less material susceptible of ethanol precipitation compared with UAE fractions and particularly the SWE fraction obtained after 15 min extraction. This fraction contained the highest polysaccharide levels yielding 15.5 g/100 g SWE fraction, levels that were higher than those obtained using other advanced technologies such as UAE after 60 min (10.1 g/100 g UAE fraction) or e.g. microwave-assisted extraction (MAE) (10.5 g/100 g MAE fraction) (Gil-Ramirez et al., 2019).

Total β -glucan content of shiitake and all generated extracts was determined using a commonly used enzymatic method (Gil-Ramirez & Soler-Rivas, 2014). The initial raw powder contained 29% (w/w) β -glucans representing the 69% of the total carbohydrates quantified being chitins in lower concentrations (5.2%). Both values were in concordance with several works (Morales, Piris et al., 2018; Morales et al., 2019; Roncero-Ramos, Mendiola-Lanao, Perez-Clavijo, & Delgado-Andrade, 2017) although they were slightly higher than others (Nitschke et al., 2011; Sari, Prange, Lelley, & Hambitzer, 2017). HWE extracted lower β -glucan amounts than UAE or SWE except for the SWEs carried out during 60 min (Fig. 2a). In fact, subcritical water extractions longer than 15 min were detrimental to get β -glucan-enriched fractions, opposite to results obtained after UAE extractions. In the latter case, 60 min extraction yielded fractions with β -glucan concentrations similar to those obtained with only 15 min SWEs. Moreover, HWE extracted from 6 to 9% of the chitins present in shiitake mushrooms and similarly UAE extracted 5–15%. Only SWE at 15 min showed the highest recovery (35%). However, since chitins are completely

insoluble in water, high recoveries were not expected. The extracted compounds might be derivative products from the chitin hydrolysis or depolymerization induced by high temperatures, pressures and/or ultrasounds as suggested in previous works (Morales, Piris et al., 2018; Morales et al., 2019; Palanisamy et al., 2014). SWEs displayed a significant decrease in chitin yield (Fig. 2b) similar to those recorded for β -glucans. It might indicate that extraction time was excessive and therefore the filters from the extraction cell were blocked and no proper extraction was made. However, it might also suggest that the excessive time at 200 °C induced hydrolysis and degradation of these two kinds of polysaccharides into lower molecular weight products that were not precipitated as polysaccharides in the analyzed extracts.

In order to test whether the different utilized extraction methodologies showed certain structure selectivity, the β -glucan contents were also evaluated using two additional methods developed to detect (1 \rightarrow 3)- or (1 \rightarrow 3),(1 \rightarrow 6)- β -glucans (Ko & Lin, 2004; Nitschke et al., 2011). The amounts of extracted β -glucans were slightly different depending on the methodology utilized e.g. after 15 min, HWE and SWE yielded fractions with similar (1 \rightarrow 3)- β -glucan concentrations (Fig. 3a), however, in the SWE fraction 84% of them were pointed as (1 \rightarrow 3),(1 \rightarrow 6)- β -glucans while the HWE fraction apparently contained only 21% of β -glucans with (1 \rightarrow 6) (Fig. 3b). Similarly, after 60 min extraction, UAE fractions showed lower (1 \rightarrow 3)- β -glucans than HWE fractions but most of them were detected as (1 \rightarrow 3),(1 \rightarrow 6)- β -glucans, reaching levels similar to SWE fractions extracted for 15 min. These results suggested that HWE was extracting more linear glucans than SWE or UAE as linear β -glucans are usually bound by (1 \rightarrow 3)- β -linkages while SWE or UAE extracted more branched (1 \rightarrow 3),(1 \rightarrow 6)- β -glucans. However, branched glucans are more soluble and therefore they are easily extractable while the linear (1 \rightarrow 3)- β -glucans remain firmly attached to the fungal cell wall, requiring stronger extraction methods. For this reason, SWE or UAE were expected to extract more linear glucans than milder treatments (HWE). An explanation for this could be that the (1 \rightarrow 3)- β -glucans obtained with HWE presented lower molecular weight being consequently more soluble than those glucans extracted with SWE or UAE (as noticed with HPSEC, see later). Moreover, a quantitative mismatch was also noticed between the values obtained with the enzymatic method calculating the total β -glucan content and the fluorimetric/colorimetric determinations. For instance, SWE (15 min) fractions contained 27.1 g/100 g total β -glucans (Fig. 2) but only 9% of them exhibited fluorescence indicating the presence of β -

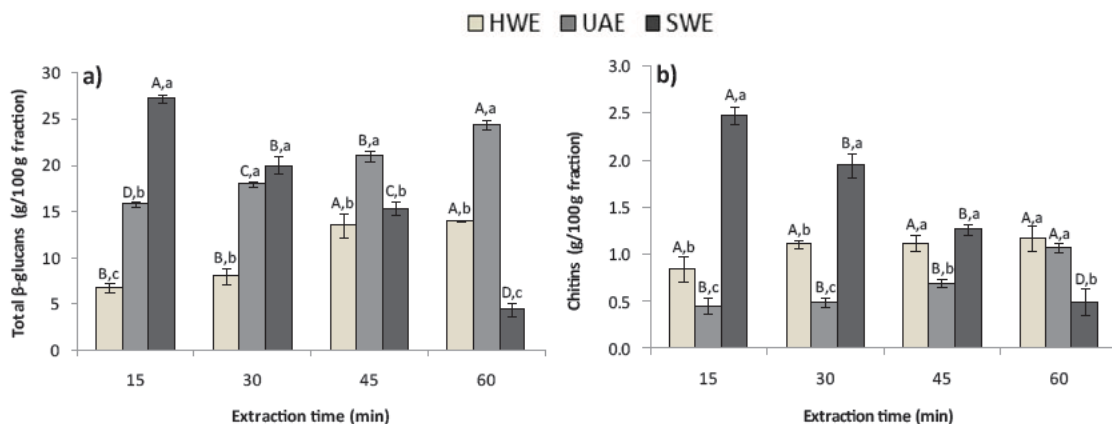


Fig. 2. Total β -glucan (a) and chitin (b) content in the fractions obtained after HWE, UAE and SWE from shiitake mushrooms. Different letters denote significant statistical differences ($P < 0.05$) between fractions obtained by the same extraction technology at different times (A-D) and obtained by different extraction technologies at the same time (a-c).

(1 \rightarrow 3) linkage (Fig. 3a) and 7% of them were detected by the colorimetric method as (1 \rightarrow 3), (1 \rightarrow 6)- β -glucans (Fig. 3b). But, according to previous publications most of the β -glucans present in shiitake mushrooms showed β -(1 \rightarrow 3), (1 \rightarrow 6) structures such as for instance lentinan (Nitschke et al., 2011) thus, these determined percentages were too low for a main component. Moreover, the SWE fraction obtained after 15 min contained only 15.5 g/100 g polysaccharides (Table 1) and 2.5 g of them were pointed as chitins (Fig. 2b). These observations might indicated that selected extraction technologies were extracting more polysaccharides than noticed or that the enzymatic determination might overestimate the total β -glucan concentration. Other reports also noticed significantly lower β -glucans values in *L. edodes* when fluorimetric methods were utilized compared to the enzymatic procedure (Gründemann et al., 2015). Therefore, the use of indirect colorimetric/fluorimetric determinations should never be considered (in complex mixtures as these extracts) as a precise determination method since their sensitivity might be influenced by the presence of many interference compounds as suggested for the fluorimetric determination (Gil-Ramirez et al., 2019). Thus, in order to define the fraction compositions more precise analytical tools were utilized.

3.2. Combining extraction methodologies

Individual extractions indicated that SWE carried out during 15 min and UAE during 60 min were adequate to extract more β -glucans than either HWE or SPE methods. However, shiitake contained approx. 34.3% polysaccharides and they were not all extracted. Thus, combination of both technologies UAE + SWE were tested to improve the β -glucan content of obtained fractions. Moreover, a pretreatment of the shiitake raw material with supercritical CO_2 was also tested because on the one hand, CO_2 generates an acid environment that might enhance cell structures breakdown and on the other hand, it extract lipid compounds that are within the mushroom dry matter (chitins and β -glucans). The latter consequence combined with the high pressure used during SFE might alter cell wall structure facilitating its disruption and the extraction of structural polysaccharides. Therefore, pretreated (SFE) material was submitted to UAE or SWE selecting the optimal extraction times.

The three tested combinations of extracting methods showed very high yields (Table 1), almost doubling those obtained with individual extractions, particularly UAE + SWE. However, not all the extracted material precipitated afterwards and they were not all polysaccharides. Combination of UAE + SWE showed results similar to SFE + SWE,

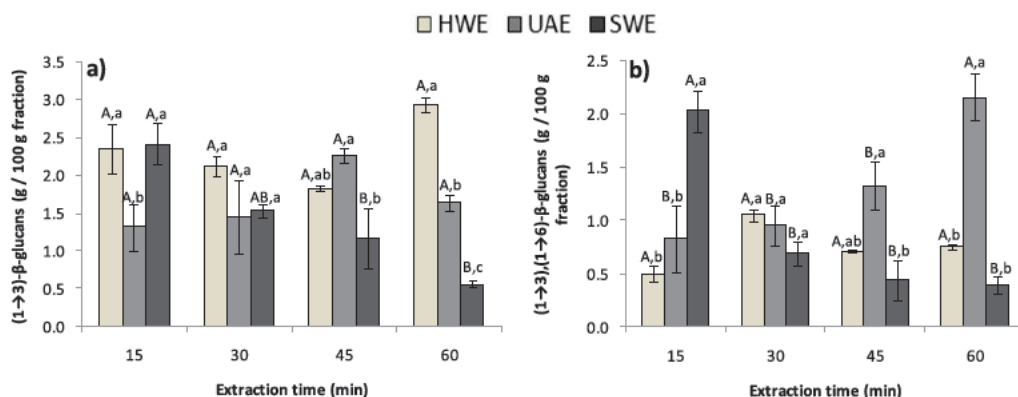


Fig. 3. Content of (a) (1 \rightarrow 3)- β -glucans and (b) (1 \rightarrow 3),(1 \rightarrow 6)- β -glucans in the fractions obtained after HWE, UAE and SWE from shiitake mushrooms. Different letters denote significant statistical differences ($P < 0.05$) between fractions obtained by the same extraction technology at different times (A-B) and obtained by different extraction technologies at the same time (a-c).

Table 2
Carbohydrates determined in shiitake powder and the fractions obtained by SPE and combined technologies (UAE + SWE, SFE + UAE and SFE + SWE). Different letters (a–d) denote significant differences ($P < 0.05$) between values of the same column.

Sample	Total carbohydrates			Total β -glucans			(1 \rightarrow 3)- β -glucans			(1 \rightarrow 3),(1 \rightarrow 6)- β -glucans			Chitins		
	g/100 g fraction	g/100 g shiitake		g/100 g fraction	g/100 g shiitake		g/100 g fraction	g/100 g shiitake		g/100 g fraction	g/100 g shiitake		g/100 g fraction	g/100 g shiitake	
Shiitake powder	–	42.10 \pm 1.17 ^b		–	28.85 \pm 0.43 ^a		–	10.40 \pm 0.22 ^a		–	3.22 \pm 0.01 ^a		–	5.19 \pm 0.13 ^a	
SPE method	15.29 \pm 3.33 ^b	5.80 \pm 1.26 ^c		6.32 \pm 0.29 ^b	2.40 \pm 0.11 ^d		2.94 \pm 0.01 ^b	1.12 \pm 0.01 ^c		0.50 \pm 0.01 ^b	0.19 \pm 0.01 ^c		1.07 \pm 0.09 ^d	0.41 \pm 0.03 ^c	
UAE + SWE	59.11 \pm 1.22 ^a	47.99 \pm 0.99 ^a		35.97 \pm 0.43 ^a	29.21 \pm 0.35 ^a		8.33 \pm 0.18 ^a	6.76 \pm 0.15 ^b		2.50 \pm 0.32 ^a	2.03 \pm 0.26 ^b		5.30 \pm 0.18 ^a	4.30 \pm 0.15 ^b	
SFE + UAE	48.92 \pm 3.43 ^a	29.22 \pm 2.05 ^b		32.17 \pm 0.28 ^a	19.22 \pm 0.17 ^c		9.09 \pm 0.94 ^a	5.43 \pm 0.56 ^b		3.03 \pm 0.24 ^a	1.81 \pm 0.14 ^b		3.86 \pm 0.05 ^c	2.31 \pm 0.03 ^d	
SFE + SWE	59.15 \pm 5.23 ^a	44.33 \pm 3.91 ^a		34.82 \pm 0.32 ^a	26.10 \pm 0.24 ^b		8.12 \pm 0.66 ^a	6.09 \pm 0.49 ^b		2.51 \pm 0.14 ^a	1.88 \pm 0.10 ^b		4.76 \pm 0.14 ^b	3.57 \pm 0.10 ^c	

apparently both pre-treatments facilitates the subsequent SWE extracting approx. 31% more polysaccharides than only SWE. SFE pre-treatment also improved approx. 41% the polysaccharide levels obtained in UAE fractions although they still contained less than UAE + SWE or SFE + SWE fractions. The combinations were more effective than SPE methods (5.1%) or other extraction technologies used to obtain polysaccharides-enriched fractions such as MAE (10.5%) (Gil-Ramirez et al., 2019).

Apparently, UAE + SWE or SFE + SWE extracted almost all the carbohydrates present in the mushroom (Table 2) and according to the enzymatic protocol they also managed to extract all the β -glucans yielding fractions containing approx. 35% β -glucans. However, the results from the fluorimetric method indicated that shiitake contained 10.4% (1 \rightarrow 3)- β -glucans and the combination of methodologies extracted slightly less compounds yielding fractions with up to 8% (1 \rightarrow 3)- β -glucans. The combination SFE + UAE was in this case more adequate to concentrate this type of compounds on fractions including up to 9% (1 \rightarrow 3)- β -glucans where one third of them were (1 \rightarrow 3),(1 \rightarrow 6)- β -glucans. In fact, the three combinations extracted similar concentrations of (1 \rightarrow 3),(1 \rightarrow 6)- β -glucans but they all generated fractions that contained more β -glucans than individual extractions. The differences between the selected combinations were more pronounced in their capacity to extract chitin-derivatives compounds. UAE + SWE extracted almost 83% of the chitins detected in the mushroom yielding fractions with more than 5% chitin-derivatives. SFE + SWE were less effective and significantly different than SFE + UAE that extracted even less derivatives. Comparing to other technologies, chitins were present in MAE extracts in approx. 2% (Smiderle et al., 2017) therefore, the pretreatment with SFE or UAE particularly before SWE seemed to be a more interesting protocol to extract β -glucans and soluble chitins-derivatives than application of individual extraction methodologies, probably because they both facilitate the disruption of the material for a subsequent subcritical water extraction.

However, similar quantitative discrepancies between the enzymatic and the fluorimetric determinations were detected as noticed within the fractions obtained with individual extractions. Thus, the enzymatic method was carried out using 100% starch and schizophyllan as respectively (1 \rightarrow 4),(1 \rightarrow 6)- α and (1 \rightarrow 3),(1 \rightarrow 6)- β -glucan standards and mixed as 50% starch:sea sand, 50% starch:schizophyllan and 50% schizophyllan:sea sand (w/w). Results indicated that, when only β -glucans were present in the mixtures, quantitative determinations were accurate (only 7% error) but, the amount of α -glucans was underestimated particularly when mixed with schizophyllan inducing a β -glucan overestimation (approx. 14.2%) since the amount of β -glucans is calculated by subtracting the α -glucan levels to the total glucan concentration. Therefore, the enzymatic, fluorimetric or colorimetric methods should be only used for preliminary assessment and more precise determinations were carried out in order to identify the compounds present in the obtained fractions.

3.3. Chemical composition of the obtained fractions

The monosaccharide composition of the polysaccharide-enriched extracts obtained with individual and combined extraction technologies was analyzed by GC–MS. Results pointed out the presence of mannose, galactose, and glucose, being the latter in high levels (Table 3). Higher extraction periods led to higher amounts of glucose in HWE extracts, while the glucose content of UAE extracts (and of the other two monosaccharides) was almost independent of the extraction time. The glucose levels were in concordance with β -glucan determinations, e.g. SWE extracted higher β -glucan amounts at 15 min than at longer extraction times as well as higher glucose levels. UAE + SWE extracts showed higher glucose levels than UAE but similar to SWE extracts suggesting that probably with the first extraction (UAE) only part of the glucans were extracted and in the second extraction an approximately 20% more was further extracted to yield the same values as with SWE

Table 3

Monosaccharide composition (%) of the precipitated extracts obtained from shiitake by the different individual and combined technologies.

Technology	Extraction time (min)	Monosaccharides (%)		
		Mannose	Glucose	Galactose
Hot water extraction (HWE)	15	21.9	68.6	9.5
	30	16.1	71.2	12.7
	45	9.3	81.5	9.2
	60	9.2	81.3	9.5
Ultrasound-assisted extraction (UAE)	15	22.3	62.0	15.7
	30	22.2	61.8	16.0
	45	22.8	62.9	14.3
	60	24.3	63.1	12.6
Subcritical water extraction (SWE)	15	10.4	83.9	5.7
	30	12.2	82.1	5.7
	45	25.0	68.5	6.5
	60	26.5	63.9	9.6
Steam Pressurized extraction (SPE)	20	13.6	74.0	12.4
UAE + SWE	60 + 15	12.2	82.8	5.0
SFE + UAE	180 + 60	11.4	81.4	7.2
SFE + SWE	180 + 15	10.0	85.3	4.7

alone. Heteropolymers including mannose and galactose in their structure are usually easily water-soluble since they are weakly attached to other molecules from the fungal matrix (Smiderle et al., 2008). HWE extracts obtained with short extraction times showed higher levels of mannose than longer extractions. On the contrary, SWE extracts (15 min) contained lower mannose and lower galactose contents, being glucose more concentrated suggesting that SWE might show certain specificity to isolate β -glucans than other heteropolymers. UAE extracts showed higher levels of mannose and galactose and lower glucose than SWE (15 min), suggesting a more heterogeneous composition than the SWE extract. The use of SFE pretreatment slightly enhanced the β -glucan selectivity noticed for SWE since SFE + SWE extracts showed a slightly higher percentage of glucose and lower galactose levels. This combination was more specific than UAE + SWE where more mannose and less glucose was noticed.

The different monosaccharide composition noticed depending on the extraction methodology utilized suggested the presence of different polymers, therefore, the extracts were also analyzed by HPSEC. Results showed heterogeneous elution profiles (Supplementary Fig. S1) confirming the presence of polysaccharides with different molecular mass. Similar profiles were observed for HWE extracts obtained at different extraction times (Fig. S1a). UAE extracts obtained after 60 min extractions were also slightly different than the other extracts obtained at shorter extraction times (Fig. S1b) but differences were more pronounced for SWE extracts. Those fractions obtained after 15 min extraction showed a peak with maximum at approx. 57 min indicating the presence of medium molecular weight compounds (Fig. S1c). With increasing extraction times, their profile shifted to other peaks with maxima approx. at 60 min indicating the presence of lower molecular weight compounds and therefore, suggesting degradation provoked by the large extraction method. UAE + SWE and SFE + SWE extracts showed a profile similar to SWE extracts while in SFE + UAE extracts seemed to increase the amount of high molecular weight compounds (R.T. approx. 45 min) compared to UAE extracts. The high molecular weight compounds noticed in UAE-related extracts were lacking in SWE extracts suggesting that the polymers extracted with SWE showed a more narrow size distribution (medium molecular weight) while UAE-related extracts contained a more heterogeneous size distribution.

Moreover, to identify the compounds present in polysaccharide-enriched extracts from the individual and combined extraction methodologies, NMR studies were carried out and compared with literature

data (Supplementary Fig. S2). The extracts contained a mixture of polysaccharides, including α - and β -glucans and a heteropolymer composed of mannose and galactose (Abreu et al., 2019; de Jesus, Smiderle, Cordeiro, de Freitas, & Iacomini, 2018; Smiderle et al., 2008). Although samples were precipitated with cold ethanol, trehalose signals were observed (δ 92.9/4.82 ppm and 92.6/4.81 ppm). Trehalose is a disaccharide, also known as mycose, and that is commonly found in large amounts in mushrooms. This sugar was also detected in *Pleurotus ostreatus* extracts obtained by SWE in previous works (Smiderle et al., 2017) and could be removed from the polysaccharide fractions after performing dialysis (data not shown).

Intense signals relative to C-1 of α -D-Glcp (δ 99.3–99.5/4.97–4.98) and to C-3O-substituted (δ 82.8–82.9/3.54–3.55) were observed, suggesting the presence of (1 \rightarrow 3)- α -glucan structures, that were also previously reported and isolated from other mushrooms such as *Fomitopsis betulina* (de Jesus et al., 2018). Moreover, the extracts showed intense ^{13}C signals arising at 102.4–103.7 ppm and ^1H signals at 4.14–4.44 ppm confirming the presence of C-1 of glucans in β -configuration (Liu et al., 2014), as well as signals at the range of δ 85.8–87.0 (^{13}C) and at δ 3.23–3.39 (^1H) related to C-3O-substituted. In addition, inverted signals at δ 68.1–68.5/3.89–3.99 and δ 68.1–68.5/3.37–3.50 confirmed CH_2 O-substitution of the same units, being in the concordance with the scientific literature, where the mainly studied glucan from shiitake mushrooms is a branched (1 \rightarrow 3)(1 \rightarrow 6)- β -glucan.

Small intensity signals of α -D-Galp were observed in almost all spectra at 98.4–98.6/4.63–4.65 ppm as well as signals of β -D-Manp at δ 101.1–101.5/4.82–4.84 ppm, being in concordance with the results of monosaccharide composition and suggesting the existence of a heteropolymer consisting of galactose and mannose.

According to these results, it was possible to observe that the polysaccharide-enriched fractions obtained from different extraction methods presented similar polysaccharide composition: (1 \rightarrow 3)- α -glucans, (1 \rightarrow 3)(1 \rightarrow 6)- β -glucans, and a heteropolymer composed of mannose and galactose. The main difference among them seemed to be the proportion of each polysaccharide extracted.

3.4. Biological activities of the obtained extracts

The production of β -glucan-enriched fractions should be encouraged for the production of functional foods because fungal β -glucans are biologically active molecules with many interesting biological activities. However, their beneficial properties can be influenced by their extraction procedure since some of them might modify the tridimensional structure of the polymer (Benito-Roman, Martín-Cortés et al., 2016). Therefore, the hypocholesterolemic and immunomodulatory activities of the precipitated extracts with the highest β -glucan contents were selected to evaluate whether after the extraction treatments, they still maintain their biological activities.

Water soluble extracts from several mushrooms including soluble β -glucans were able to inhibit the key enzyme of the cholesterol metabolism (Gil-Ramírez et al., 2017), therefore, obtained extracts were tested as HMGR (3-hydroxy-3-methylglutaryl coenzyme A reductase) inhibitors. Results indicated that SFE + UAE and SFE + SWE extracts showed inhibitory capacity similar to 0.5 mg/mL pravastatin, the statin utilized as positive control (Table 4). The UAE + SWE extract exhibited a slightly lower capacity. The combination of these two extraction methodologies seemed to generate extracts with lower inhibitors concentration or to be slightly detrimental for the structure of the HMGR inhibitors but still, it was better combination than HWE where half of the inhibitory activity was lost. Gil-Ramírez, Clavijo et al. (2013) studied the HMGR inhibitory activity of SWE extracts obtained from *L. edodes* and indicated that extracts obtained at 150 °C (5 cycles 5 min) showed less inhibitory activity than similar extracts obtained at 25 °C probably because the inhibitors were thermal sensitive. However, SFE + SWE or UAE + SWE extracts still retained very high inhibitory

Table 4

HMGR inhibitory activity (%) of the obtained precipitated extracts obtained by HWE during 60 min (HWE60') and combination of advanced methodologies. Different letters (a–c) denote significant differences ($P < 0.05$) between samples.

Sample	HMGR inhibition (%)
HWE60'	42.52 \pm 0.01 ^c
UAE + SWE	84.41 \pm 6.98 ^b
SFE + UAE	89.03 \pm 0.48 ^{ab}
SFE + SWE	87.32 \pm 1.89 ^{ab}
Pravastatin	99.21 \pm 0.36 ^a

activity. Perhaps, the pre-treatment with UAE or SFE prior to SWE protected/modulated the inhibitors structure making them more active or less susceptible to thermal degradation or perhaps, the *L. edodes* strain was different than the one utilized in the previous work as differences between varieties or cultivation methods seemed to influence their inhibitory properties too (Gil-Ramirez, Clavijo et al., 2013).

The immunomodulatory activities of the extracts obtained with combined methodologies were also tested as their capacity to reduce the secretion of pro-inflammatory cytokines in macrophages differentiated from THP-1 human monocytes cell line. The preliminary experiments to assess the extract cytotoxicity indicated that when applied

up to 200 $\mu\text{g/mL}$ viability of THP-1 macrophages was not affected (data not shown). Thus, the immunomodulatory activity was tested in two subtoxic concentrations (100 and 200 $\mu\text{g/mL}$). The THP-1 macrophages stimulated with LPS (positive control) exhibited a significant release of the three pro-inflammatory cytokines studied (1295 pg/mL TNF α , 4586 pg/mL IL-1 β and 1837 pg/mL IL-6) compared to non-stimulated cells (negative control) (Fig. 4). Addition of the extracts obtained with combined methodologies significantly reduced the amount of TNF α liberated in the media. When applied at the highest tested concentration a 24–32% reduction in TNF α was noticed (down to 883 pg/mL in the case of SFE + UAE), however, the extract obtained after 60 min HWE did not effectively modulate any response. SFE + UAE seemed to be more effective because it induced the mentioned TNF α reduction and was the only extract inducing a 25% reduction (3462 pg/mL) in IL-1 β secretion when applied at 200 $\mu\text{g/mL}$. The IL-6 release was also inhibited approx. 25% by SFE + UAE and SFE + SWE (1420 and 1343 pg/mL, respectively) extracts being the latter also significantly effective when applied at lower concentration. However, SWE extracts obtained from *L. edodes* at 50 °C (5 cycles 5 min) reduced approx. 90% the release of IL-6, IL-1 β and 20% TNF α while if the temperature was higher (200 °C) only a slight reduction in IL-6 was noticed (Gil-Ramirez, Lopez de las Hazas et al., 2013). Thus, the temperature utilized in the production of the SFE + SWE extract could have impaired their beneficial immunomodulatory properties. Thus, SFE + UAE was a more adequate combination of methodologies to maintain the

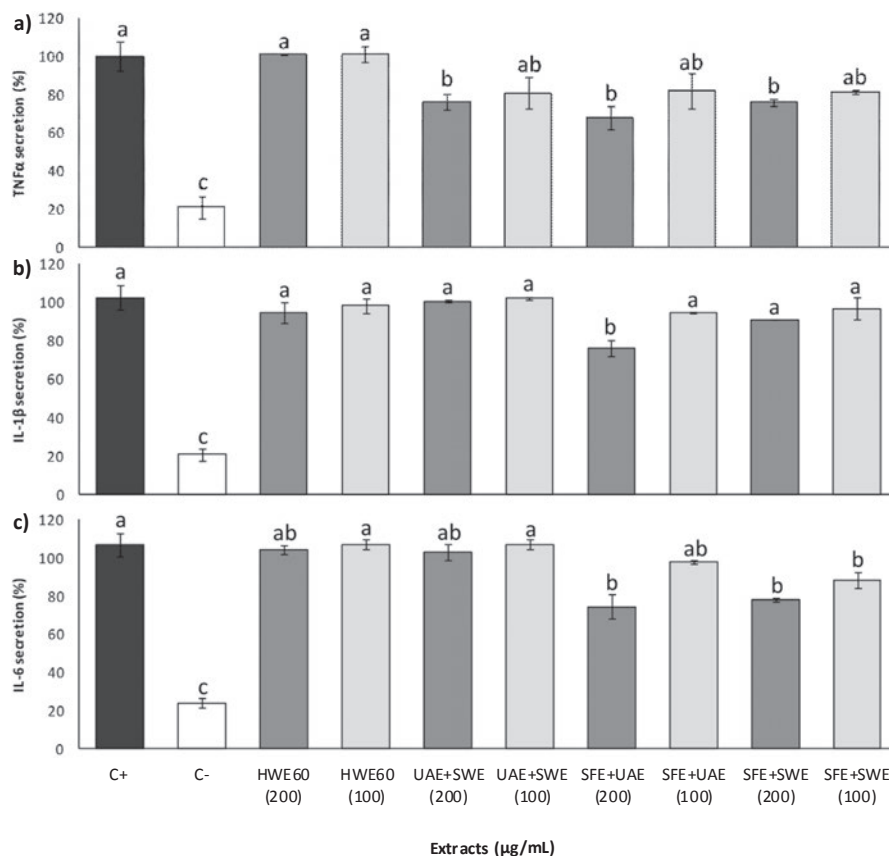


Fig. 4. Levels of (a) TNF α , (b) IL-1 β and (c) IL-6 secreted by THP-1/M activated with LPS in presence of HWE (60 min), UAE + SWE, SFE + UAE and SFE + SWE extracts. Positive control: cells stimulated with LPS but in absence of extract. Negative control: non LPS-activated cells. Different letters (a–c) denote significant differences ($P < 0.05$) between samples.

immunomodulatory properties since its extract could reduce the liberation of all pro-inflammatory cytokines tested but, their activity might be influenced by the temperature utilized.

4. Conclusion

In conclusion, submission of shiitake mushrooms to UAE or SFE followed by UAE or SWE were more effective methods to obtain β -glucan-enriched fractions than individual UAE or SWE extractions. The generated fractions contained approx. 20% polysaccharides although according to enzymatic determinations they included approx. 34% β -glucans. Fluorimetric/colorimetric methods pointed out lower amounts of (1 \rightarrow 3) and (1 \rightarrow 3),(1 \rightarrow 6) linked β -glucans. These discrepancies might be caused because they were determined by indirect rough methods that should be considered as preliminary estimations. More accurate analytical methods indicated that UAE fractions contained more heterogenic composition (more mannose and galactose and less glucose contents) and polymers of higher molecular weight than SWE (15 min) or SFE + SWE fractions. UEA-related extracts (e.g. 60 min or SFE + UAE, etc.) also extracted lower chitin-derivative contents than SWE-related extracts (15 min, UAE + SWE, SFE + SWE, etc). However, the main differences among the fractions seemed to be the extracted polysaccharides ratio since they all contained (1 \rightarrow 3)- α -glucans, (1 \rightarrow 3),(1 \rightarrow 6)- β -glucans, and heteropolymers composed of mannose and galactose. Moreover, the obtained fractions might be of interest to design functional foods with hypocholesterolemic properties since they showed high HMGR inhibitory activity, even higher than other reported extracts obtained at high temperatures similar to those used in SFE + SWE or SFE + UAE (Gil-Ramirez, Clavijo et al., 2013). But, the immunomodulatory properties of the extracted β -glucans might be compromised because although they were still able to reduce secretion of TNF α , IL-1 β and IL-6 in macrophage cell lines, only the SFE + UAE fractions managed to reduce them approx. 20% while other extracts obtained using SWE at lower temperatures were reported more effective (Gil-Ramirez, Lopez de las Hazas et al., 2013).

Ethics statement

Our research did not include any human subjects or animal experiments.

We have read and adhere to the Publishing Ethics.

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Declaration of Competing Interest

None.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jff.2019.103446>.

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Production of a β -D-glucan-rich extract from Shiitake mushrooms (*Lentinula edodes*) by an extraction/microfiltration/reverse osmosis (nanofiltration) process

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ABSTRACT

A pilot-scale process combining extraction of Shiitake mushroom (*Lentinula edodes*) powder in water (98 °C, 1 h), cross-flow microfiltration and reverse osmosis (nanofiltration) was performed to obtain β -glucan-rich extracts. Suspensions (45–80 L) obtained after 3 extractions were clarified by microfiltration reducing their turbidity to < 1 NTU. Membrane flux was completely recovered after filtration. One of the clarified extracts was concentrated (to 6–7 L) by reverse osmosis (Nanomax95) and the other two by nanofiltration (Nanomax50 and ALNF99-2517). Different physicochemical parameters (permeate flux, total soluble substances, total suspended particles and electrical conductivity) were monitored during filtration and the bioactive compounds present in the obtained fractions (β -glucans, total carbohydrates, chitins, eritadenine, lenthionine, ergosterol) were analyzed. The more adequate membrane for Shiitake extract concentration was Nanomax50 because it showed higher filtration flux and higher values of bioactive compounds in the obtained extract than the extracts obtained with the other two membranes.

Industrial relevance: This work describes a pilot-scale procedure for obtaining β -D-glucan-rich extracts from *Lentinula edodes* (Shiitake mushrooms). The extracts might be used in novel functional foods due to their high content in hypocholesterolemic compounds. The process combines extraction with boiling water, cross-flow membrane clarification and reverse osmosis/nanofiltration concentration of β -D-glucans. The procedure is scalable to industrial level.

1. Introduction

β -D-Glucans are polysaccharides consisting of D-glucose units linked by β -glycosidic bonds which can be found in several natural sources such as yeasts, bacteria, algae, cereals and mushrooms (Kagimura, da Cunha, Barbosa, Dekker, & Malfatti, 2015; Zhu, Du, Bian, & Xu, 2015). They have attracted attention due to their biological activities, including immune-modulating, anti-tumour, antioxidant and anti-inflammatory properties (Arena et al., 2016; Meng, Liang, & Luo, 2016; Smiderle et al., 2013; Vamanu, 2012). Furthermore, several studies indicated that β -D-glucans are also able to lower serum cholesterol and blood glucose (Gil-Ramirez et al., 2016; Kim, Kim, Chouli, & Lee, 2005; Palanisamy et al., 2014; Tong et al., 2015).

Different extraction procedures at lab, pilot or industrial scale have been described and adapted depending on the source and nature of the β -D-glucans and most of them are based on hot water extractions. However, sometimes their proper solubilization requires more aggressive conditions: alkaline or acidic mediums, ultrasound, microwave and enzymes aids or temperatures higher than 100 °C and these treatments might also contribute to their partial degradation or modification of their native structure altering their biological activities (Anguilo-Aguayo, Walton, Vinas, & Tiwari, 2017; Immerstrand et al., 2009; Ruthes, Smiderle, & Iacomini, 2015; Smiderle et al., 2017).

After extraction, several purification steps are required to concentrate β -D-glucans, usually by removing other substances. One of the most used, simple and effective procedure for separation of β -D-glucans

Abbreviations: MF, microfiltration; NF, nanofiltration; RO, reverse osmosis; TSS, total soluble substances; TSP, total suspended particles; E, extract; ES, extraction series; P_{TM} , trans-membrane pressure

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from other molecules (particularly from low molecular mass compounds) is their precipitation with ethanol (Ruthes et al., 2015), although other solvents such as ammonium sulfate or acetone solutions have also been used (Irakli, Biliaderis, Izydorczyk, & Papadoyannis, 2004; Shi, 2016). Other purification techniques are column-based fractionation procedures such as size exclusion, anion-exchange or affinity chromatography (He et al., 2017; Yoshida, Honda, Tsujimoto, Uyama, & Azuma, 2014; Zhang, Zhou, Yang, & Chen, 1998), but they are useful mainly for preparative separation at lab scale. For purification of larger extract volumes, membrane separation processes such as ultrafiltration and diafiltration have been efficiently used to purify β -D-glucans and other polysaccharides from mushrooms and other food (Amaral et al., 2008; Benito-Roman, Alonso, Palacio, Pradanos, & Cocero, 2014; Bhanja et al., 2012; Carbonero et al., 2012).

In this study, Shiitake mushroom (*Lentinula edodes*) powder was submitted to extraction in boiling water to obtain a β -D-glucans enriched extract with beneficial properties for cardiovascular health. A double stage cross-flow microfiltration and reverse osmosis/nanofiltration was used to remove suspended particles and to increase β -D-glucan concentration. Filtration was carried out using different membranes and some physicochemical parameters of the concentrate and permeate streams were evaluated during the filtration (total water soluble substances, turbidity and electrical conductivity) to define the optimal treatment. Furthermore, β -D-glucan content was determined in the obtained extracts and after each purification step as well as other important compounds of Shiitake mushrooms with hypocholesterolemic activities such as eritadenine, ergosterol and chitins (as source to generate chitosan).

2. Materials and methods

2.1. Materials and reagents

Fine powder (particle size < 0.5 mm, moisture < 5%) of *Lentinula edodes* S. (Berkeley) fruiting bodies was purchased from Glucanfeed S.L. (La Rioja, Spain) and stored in darkness at -20°C until further use.

Hexane (95%), chloroform (HPLC grade), methanol (HPLC grade) and acetonitrile (HPLC grade) were obtained from LAB-SCAN (Gliwice, Poland) and absolute ethanol, sodium carbonate (Na_2CO_3) and sulfuric acid (H_2SO_4), from Panreac (Barcelona, Spain). Potassium hydroxide (KOH), ascorbic acid, 2,6-Di-*tert*-butyl-*p*-cresol (BHT), bovine serum albumin (BSA), Bradford reagent, acetylacetone, *p*-dimethylamine-benzaldehyde, Tris (Trizma®) base, hydrochloric acid (37%), trifluoroacetic acid (99%), phenol, as well as, hexadecane, ergosterol (95%), D-glucose and D-glucosamine hydrochloride were purchased from Sigma-Aldrich Quimica (Madrid, Spain). β -glucan Assay Kit Megazyme® was acquired from Biocon (Barcelona, Spain), D-eritadenine (90%) from Sy Synchem UG & Co. KG (Felsberg, Germany) and lenthionine (80%) from Cymit (Barcelona, Spain). All other reagents and solvents used in this study were of analytical grade. MilliQ-grade water was produced in a MilliQ® Integral 3 purification system (Merck Millipore, Billerica, USA); demineralized water with electrical conductivity of $7\text{--}8\ \mu\text{S}/\text{cm}$ was obtained by a reverse osmosis (RO) unit (Genius 300, Filtec Depuradoras, Girona, Spain).

2.2. Pilot scale solid/liquid extraction unit

Large amounts of Shiitake extracts (45–80 L) were prepared using a pilot-scale solid/liquid extraction unit, provided with a 30 L extraction vessel and a thermostatic system, allowing temperatures maintenance from 30 to 99°C . A Nylon mesh strainer bag with a mean pore size of $30\ \mu\text{m}$ was used to hold the suspension in the extraction vessel. Homogenization of the suspension and intensification of the matter transfer from the solid to the liquid phases were carried out by mechanical agitation of the suspension inside the strainer bag.

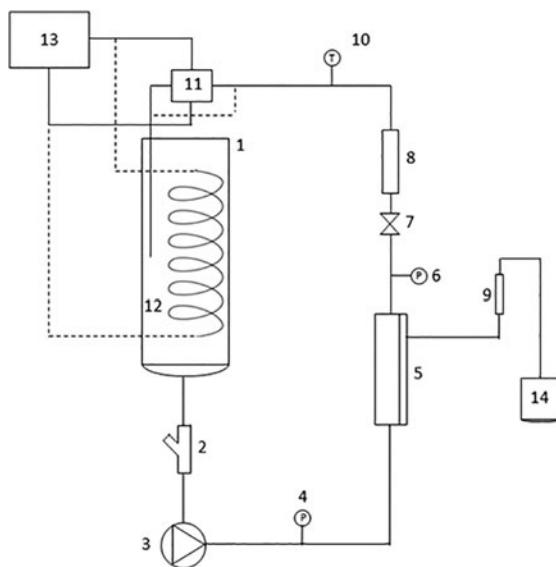


Fig. 1. Scheme of multifunctional pilot pressure-driven cross-flow membrane device: (1) feed tank, (2) strainer, (3) pump, (4) inlet pressure gauge, (5) membrane housing, (6) outlet pressure gauge, (7) pressure control valve, (8) concentrate flowmeter, (9) permeate flowmeter, (10) thermometer, (11) plates and frames heat exchanger, (12) tubular spiral wound heat exchanger, (13) chiller and (14) permeate recipient vessel.

2.3. Pilot scale pressure-driven crossflow membrane unit

All treatments for clarification and concentration of the Shiitake extracts were carried out by a self-designed multifunctional pilot pressure-driven crossflow membrane unit (Fig. 1), provided by a 20 L feed tank (1), 0.25 mm mesh stainless still Y-type strainer (2), variable flow ($0\text{--}2500\ \text{L}/\text{h}$)/high pressure (up to 50 bar) membrane pump (Hydra-Cell G-25, Wanner Engineering Inc., Minneapolis, USA) (3), inlet pressure gauge ($0\text{--}6$ or $0\text{--}60$ bar) (4), membrane housing (5), outlet pressure gauge ($0\text{--}6$ or $0\text{--}60$ bar) (6), needle pressure control valve (7), online concentrate rotary flowmeter (8), online permeate rotary flowmeter (9), online thermometer (10), online plates and frames (11) or immersed tubular spiral wound (12) heat exchangers, chiller (13) and permeate recipient vessel (14). Depending on the used membranes and housings, the unit has a dead volume of 6 to 7 L. All tubing's, vessels and parts of the measurement instruments which were in contact with the fluid were from AISA 316 and 316L stainless still. Pump membranes were of food grade ethylene propylene diene monomer (EPDM) and the mobile parts of the tubing's, from silicone.

2.4. Lab-scale extraction of β -D-glucans from Shiitake mushroom powder

Temperatures of 60 and 98°C and extraction times of 1, 2 and 3 h were tested at lab scale, according to the following procedure: mushroom powder (10 g) was placed into 0.5 L round flasks and 300 mL MilliQ-grade water was added to each of them ($33.3\ \text{g}/\text{L}$). The mixtures were heated at the respective temperatures and stirred in a thermal magnetic plate (with reflux for extractions at 98°C). Furthermore, the same amounts and ratio of mushroom powder and water were autoclaved at 120°C during 20 min. The soluble fractions were clarified by centrifugation (7000 rpm, 7 min) and freeze-dried. In addition, extractions were performed again and polysaccharides were precipitated by adding 3 volumes of ethanol, mixing vigorously and keeping the mixtures overnight at 4°C . Later on, the samples were centrifuged

Table 1

Amounts of solvent (water), substrate (Shiitake powder), substrate to solvent ratio and number of extraction batches used in the three extraction series (ES1, ES2, ES3) of β -D-glucans from Shiitake mushrooms at pilot plant conditions.

Extraction series	Water used for extraction (L)	Shiitake powder used for extraction (kg)	Substrate to solvent ratio (g/L)	Number of extraction batches (–)
ES1	60	1.5	50.0	2
ES2	90	2.0	66.7	3
ES3	120	1.8	60.0	4

(10,000 rpm, 15 min) and the precipitates lyophilized. Moreover, different substrate to solvent ratios were tested apart from 33.3 g/L such as 50, 66.7, 133.3 and 200 g/L at 98 °C during 1 h. All extractions were carried out in duplicate.

2.5. Pilot-scale extraction of β -D-glucans from Shiitake mushroom powder

After optimization of the extraction parameters at lab scale, three series of extractions (ES1, ES2 and ES3) were carried out at pilot scale in order to adequate some extraction parameters, such as processing time, suspended particle load, β -D-glucan concentration and batch volumes to the capacities and limitations of the membrane separation equipment. The studied parameters are shown in Table 1.

Extraction series 1 (ES1) reproduced directly the optimal conditions obtained at lab scale. ES2 and ES3 were carried out to improve the extraction efficiency and adequate the extract volumes to the trans-formation capacity of the microfiltration (MF) unit.

In all cases, demineralized water (30 L) was heated up to 85 °C. Shiitake powder (Table 1) was introduced into the Nylon mesh strainer bag and immersed into the hot water with intense agitation in order to disperse the solid phase into the liquid phase and inhibit the action of polyphenol oxidases naturally present in Shiitake mushrooms. Then, the suspensions were heated up to 98 °C and maintained at this temperature for 1 h. Extractions were ended by draining the liquid phase from the extraction chamber. The insoluble particles that remained in the strainer bag were frozen and freeze-dried. The cloudy extract was left to settle overnight at 4 °C and for ES2 and ES3, the clear phase was separated by racking and stored at 4 °C for the next fine clarification by MF (ES1 was submitted to MF without racking). The sediments were centrifuged and the solid phase was freeze-dried and added to the insoluble solids. The supernatant was added to the settled extract and submitted to the next MF process.

2.6. Fine clarification of Shiitake extracts by cross-flow membrane microfiltration (MF)

Fine clarification of the cold settled Shiitake extracts was carried out by the above described pressure-driven membrane unit (Fig. 1), set at the low-pressure mode. In this case the equipment includes a tubular spiral wound heat exchanger (12) (immersed into the feed tank (1)), inlet and outlet pressure gauges (4 and 6), sensitive in the interval of 0–6 bar and a multichannel ceramic membrane from CeraMem Corporation (Waltham, MA, USA) (Table 2). Prior to use, the membrane

was conditioned by washing with demineralized water. Initial (L_p^0) and post-regeneration (L_p^R) membrane hydraulic permeability was determined by plotting the water flux values at different transmembrane pressures (P_{TM}) at temperature of 20 ± 2 °C. Pressure was fixed by varying the inlet flow rate at completely open pressure control valve. The same measurements were carried out with the Shiitake extract at the same operating conditions.

Fine clarification was carried out by filtration of the extracts in a concentration mode at 0.7 bar constant P_{TM} and 20 °C. When 5 L of permeate were collected, 5 L of the initial cold settled extract were added into the feed tank all over the treatments. Concentration volumetric factors (F_C) for each treatment were calculated as: $F_C = V_{initial} / V_{final}$, where $V_{initial}$ was the initial volume of the extract and V_{final} was the final volume of the corresponding concentrate. Filtration was stopped when the whole volume of extract was processed. The highly dense concentrate (approx. 7 L) was discarded. Samples (5 mL) were collected during filtration from both permeate and concentrate streams and analyzed for their content of total soluble substances (TSS or °Brix) and turbidity.

2.7. Concentration of Shiitake extracts by nanofiltration (NF)/reverse osmosis (RO)

Concentration of the fine clarified Shiitake extracts was carried out by the already described pressure-driven membrane unit (Fig. 1) set at the high-pressure mode. In this case, plates and frames heat exchanger (11) and high pressure (0–60 bar) inlet and outlet pressure gauges were used. Three spiral wound membranes, Nanomax95 and Nanomax50 from Millipore (Bedford, MA, USA) and ALNF99-2517 from Alfa Laval (Lund, Sweden) (Table 2) were tested. Prior to use, the membranes were conditioned by washing with demineralised water. Initial (L_p^0) and post-regeneration (L_p^R) membrane hydraulic permeability was determined as described earlier for the MF membrane. The feed flow rates (Q_F) for Nanomax95 and Nanomax50 membranes were set at 600 and 800 L/h respectively, whereas those for the Alfa Laval's membrane was 1100 L/h. Pressure was fixed by appropriate adjusting of the pressure control valve. The same measurements were carried out with the Shiitake extract, at the same operating conditions.

Processing was carried out by filtration of the extracts in a concentration mode at 24–29 bar P_{TM} and 20 °C. Operational P_{TM} were determined from the corresponding plots of filtration flux (J) = $f(P_{TM})$. In general, they were selected as the highest value from the linear response of each membrane to the corresponding extract. Thus, the extracts from the ES1, ES2 and ES3 series were concentrated by Nanomax95, Nanomax50 and ALNF99-2517 at P_{TM} of respectively 24, 29 and 25 bar. When 5 L of permeate were collected, 5 L of clarified extract were added into the feed tank, all over the treatments. F_C 's for each treatment were calculated as described already for the microfiltration process. Filtration was stopped when the whole volume of extract was processed. The concentrate was drained from the equipment, freeze-dried and stored at 4 °C for further analysis as well as aliquots of 5 L of the 3 permeates. Samples of 5 mL were collected during filtration from both, permeate and concentrate streams and analyzed for their content of total soluble substances (TSS), turbidity, pH and electrical conductivity.

After filtration, membranes were cleaned by consecutive washings

Table 2

Technical characteristics of the used membranes (MF: microfiltration, NF: nanofiltration, RO: reverse osmosis).

Membrane designation	Housing model	Nominal pore size (μ m)	NaCl rejection (%)	MgSO ₄ rejection (%)	Filtration surface (m ²)	Membrane material	Mode of operation
LM-0500-M	Not available	0.5	–	–	0.13	Mixed oxides	MF
Nanomax95	Helicon-RO4	–	94	97	0.37	Polyamide	RO
Nanomax50	Helicon-RO4	–	65	96	0.37	Polyamide	NF
ALNF99-2517	M2.5-PN64	–	–	≥ 98%	1.0	Composite	NF

with water at room temperature for 15 min, followed by water at 45 °C for 15 min and chemical regeneration with 0.5 M NaOH solution at 50 °C during 60 min for the MF membrane and 0.1 M NaOH solution at 45 °C for 60 min for the NF and RO membranes. Final washing with water until both rejection and permeate streams became neutral (pH 7) at room temperature was carried out for all membranes before conservation.

Retention coefficients (R) were calculated for each measured parameter, according to $R = (1 - C_p / C_f) \cdot 100$ (%), where C_p and C_f are the concentration of each measured parameter in the permeate and the feed streams, respectively.

2.8. Physicochemical analyses

Total soluble substances (TSS) of the extracts were measured directly by hand-held refractometer Atago in the interval of 0 to 32% (°Brix). Total suspended particles (TSP) were estimated indirectly by measuring turbidity with a TN100 infrared turbidimeter (ThermoFisher Scientific, Spain), attuned for measurements in the interval of 0 to 800 NTU (nephelometric turbidity units). Samples with turbidities higher than 800 NTU were diluted appropriately with water to enter in this interval and values were calculated according to the number of dilutions. Electrical conductivity was determined by direct measurement of samples by a GLP 32 model conductivity meter from Crison Instruments (Barcelona, Spain).

2.9. Chemical composition analyses

β -D-Glucan content was measured using a mushroom and yeast specific β -glucan kit (β -glucan Assay Kit Megazyme®) following the instructions of the user's manual and as described in Palanisamy et al., 2014.

Total carbohydrate content was determined by the phenol-sulfuric acid method, adapted from Dubois, Gilles, Hamilton, Rebers, and Smith (1956), as described in Fox and Robyt (1991).

Chitin content was determined by a colorimetric method based on Smiderle et al. (2017). For this, the sample (5 mg) were hydrolyzed with 6 M HCl at 100 °C for 2 h and adjusted to pH10.0 after cooling down. The hydrolyzed sample (250 μ L) was used for the colorimetric method according to Rementeria et al. (1991). Samples were read at 530 nm using an Evolution 600 UV-VIS (Thermo Fisher Scientific, Spain) spectrophotometer and glucosamine hydrochloride was used to prepare the standard curve.

Sterols were extracted from Shiitake powder and its soluble and insoluble parts, using the equipment and following the procedure described by Gil-Ramirez et al. (2013). Ergosterol was used as standard to develop and validate the GC method and hexadecane (10% v/v) as internal standard.

Eritadenine was extracted from the samples following the procedure of Afrin, Rakib, Kim, Kim, and Ha (2016) with some modifications. Briefly, samples (1 g) were mixed with 10 mL of 60% ethanol (v/v) and stirred for 2 min. The mixtures were centrifuged (15 min, 7000 rpm, 10 °C) and the supernatants were collected by decantation. Afterwards, aliquots of 10 mL of 60% ethanol (v/v) were again added for a second extraction and both supernatants were pooled together and submitted to vacuum filtration. The filtrate was concentrated on a rotary vacuum evaporator at 60 °C until dryness. Identification and quantification of eritadenine were carried out using a C18 Spherisorb ODS2, 250 \times 4 mm i.d. analytical column with a 5 μ m particle size (Waters, Mississauga, Ontario, Canada), coupled to an HPLC system (Pro-Star 330, Varian, Madrid, Spain) with photodiode array detector (PAD). Samples were dissolved in water:acetonitrile (98:2, v/v 1% TFA) used as mobile phase (5 mg/mL) and aliquots of 10 μ L were injected and developed at constant flow (0.5 mL/min). Eritadenine was identified and quantified at 260 nm using a commercial standard showing its characteristic UV-spectra and retention time (11.6 min).

Lenthionine extraction was carried out according to the procedure of Hiraide, Kato, and Nakashima (2010) with slight modifications. Basically, samples were mixed with 0.2 M Tris-HCl buffer pH 8.0 (50 mg/mL) and stirred for 1 h. Afterwards, 0.5 mL methanol was added and the mixture was stirred and centrifuged (14,000 rpm, 5 min). Supernatant was collected and pooled together with the others obtained after repeating this step three times. Later on, MilliQ water (2.5 mL) was added to the supernatants and filtered through a PVDF filter (0.45 μ m). The solution was introduced into a 50 mg ODS Sep-Pack cartridge (Waters, Mississauga, Ontario, Canada) preconditioned according to the manufacturer instructions. The cartridge was washed with 1 mL of 30% methanol (v/v) and lenthionine was eluted with 1 mL of 65% methanol (v/v). Finally, the eluate (50 μ L) was injected into an HPLC-PAD system (same column and equipment than previously described for eritadenine analysis) to identify and quantify lenthionine (retention time 10.4 min) at 230 nm using an isocratic mobile phase of 65% methanol, constant flow (0.7 mL/min) and 45 °C column temperature. Commercial lenthionine was used as a reference substance.

3. Results & discussion

3.1. Extraction of β -D-glucans at lab scale

Lab scale preliminary β -D-glucan extractions were carried out to figure out optimal extraction temperature, time and substrate to solvent ratio. Results indicated that the extraction yields of total polysaccharides were rather similar under most of the studied conditions (Fig. 2a). Extraction at 98 °C for 3 h showed the highest yield (5% w/w) being similar to those found by Smiderle et al. (2017) and Angulo-Aguayo et al. (2017) using respectively microwaves, pressure and heat at 50 °C during 5 min and ultrasounds during 15 min. However, stronger heat exposure (> 2 h at 98 °C and only 20 min at 120 °C, Fig. 2b) was detrimental for β -D-glucan content, suggesting that 98 °C

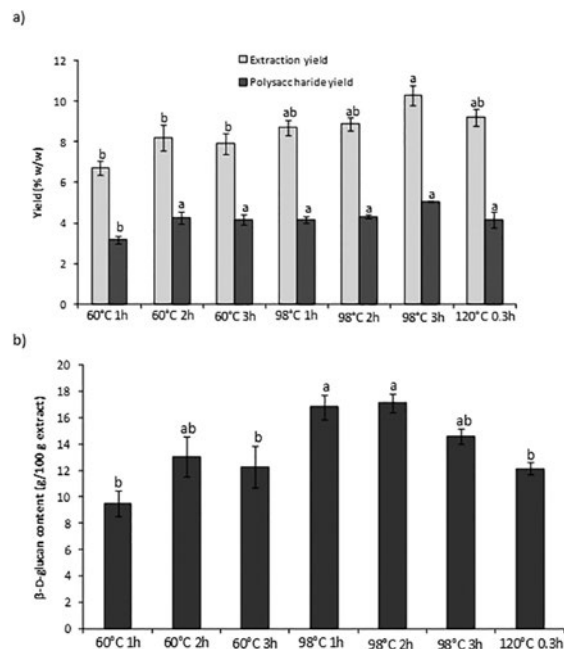


Fig. 2. Dry matter, total polysaccharide (a) and β -D-glucan (b) extraction yields obtained after extraction of Shiitake powder at lab scale at different temperatures and time (% w/w of initial dry matter). Different letters (a–b) between values of the same series denote significant differences ($P < 0.05$).

Table 3

Whole extract volume, turbidity (before and after cold clarification), total soluble substances (TSS) and β -D-glucan content (dry weight) of the Shiitake mushroom extracts (E1, E2 and E3) obtained after the three extraction series (ES1, ES2, ES3) at pilot plant conditions.

Extraction series	Whole extract volume (L)	Turbidity before fine clarification (NTU)	Turbidity after fine clarification (NTU)	TSS content (%)	β -D-Glucan content (%)
ES1	45	1407	0.28	1.9	17.8
ES2	55	1832	0.28	4.2	13.2
ES3	80	1213	0.64	2.9	13.4

and 1 or 2 h were more suited for the extraction of soluble β -D-glucans.

In addition, different substrate to solvent ratios (33, 50, 67, 133 and 200 g/L) were tested and no significant differences between extraction yields were found (data not shown) but ratios over 50 g/L hindered the separation of the liquid extract from the insoluble fraction because of the clogging of the Nylon bag cloth. Therefore, a ratio of 50 g/L substrate to solvent was selected for the first extraction series (ES1) at pilot scale.

3.2. Extraction of β -D-glucans at pilot scale

Pilot-scale extractions were carried out to define processing time, suspended particle load, β -D-glucan concentration and batch volumes prior to subsequent membrane separation processes.

Firstly, direct up scaling of the optimal lab conditions were tested: substrate to solvent ratio was 1.5 kg Shiitake powder/30 L water/batch and they were macerated at 98 °C during 1 h (ES1, Table 1). Two extraction batches were carried out at these conditions and a total volume of 45 L of extract (E1) was obtained containing 1.9% TSS, 17.8% β -D-glucans and 1407 NTU turbidity (Table 3). Then, the obtained β -glucan-rich extract was clarified by MF in one batch for a reasonable time of < 8 h, at relatively low P_{TM} (0.7 bar) (Fig. 4) and without any technical or operational problems. These results suggested that improving of the β -D-glucan yield and increasing of the processing time were possible.

In the second extraction series (ES2) an increase of the substrate to solvent ratio of 50 g/L to 66.7 g/L and the number of extraction batches from 2 to 3 was carried out. Nevertheless, these changes led to an important decrease of the permeate flux (Fig. 4) and increase of the TSP content (Table 3), which was detrimental for the MF equipment as some part of the suspended particles tended to decant and obstruct the equipment piping and connections, leading to breaking of the process and a need for dilution of the extract to reestablish the filtration process. However, the membrane resisted this heavy operational conditions and the whole volume of Shiitake extract was processed in only one batch, without any physical or chemical cleaning of the membrane. These outcomes evidenced that substrate to solvent ratio of 66.7 was too high for further treatments, but that the volume of extract might be still increased.

Thus, the last extraction series (ES3) was carried out at a substrate to solvent ratio of 60 g/L (Table 1). Four extraction batches of 30 L were sequentially carried out and 80 L of total extract (E3) were produced, containing 1213 NTU turbidity, 2.9% TSS and 13.4% β -D-glucans (Table 3). Filtration of an extract with such physicochemical characteristics allowed the establishment of very good filtration flux of 70–60 L/hm² (Fig. 4) at relatively low P_{TM} (0.7 bar) in the MF process and to transform 80 L of extract in one MF batch (without intermediate discharge of concentrate) and without any technical or operational problem. Nevertheless, the transformation of 80 L of extract would require a change of 2 turn's regime of staff to cover an interval of 20 h of working time.

Results of the β -D-glucans analysis showed that the extract obtained

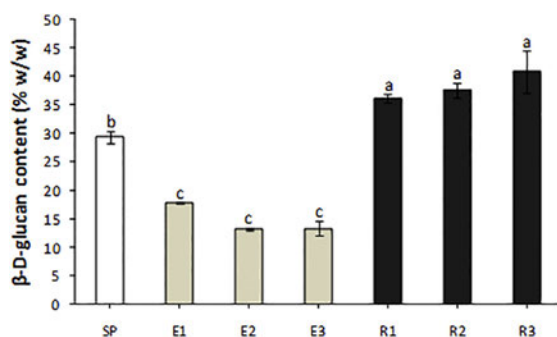


Fig. 3. β -D-Glucan content (% w/w) of raw Shiitake mushroom powder (SP), soluble extracts (E1, E2, E3) and insoluble fractions (R1, R2, R3) from the three studied extraction series (ES1, ES2, ES3). Different letters (a–c) denote significant differences ($P < 0.05$) between values of the same series.

at the lowest substrate to solvent ratio (E1) had the highest concentration of soluble β -D-glucans, 17.8% (Fig. 3). This value was very similar to the obtained at the same conditions at lab scale. Nevertheless, in all cases, the content of soluble β -D-glucans was considerably lower than those of the initial raw Shiitake powder (SP) (29%) and the insoluble material (R1–R3) (36–41%), indicating the existence of a considerable quantity of β -D-glucans that could not be extracted by the studied extraction conditions. These results suggest that other techniques, such as alkaline or ultrasound- or microwave-assisted or steam explosion extraction should be considered if higher yields of soluble β -D-glucans are required. However, taking into account costs for investments and scaling to pilot equipment for production of larger extract amounts, simple solid/liquid extraction with boiling water is still one of the most technically and economically viable alternatives.

Aliquots of soluble and non-soluble extracts were submitted to centrifugation to observe differences in the content of β -D-glucans, but no significant differences were found (data not shown) so this centrifugation step was not included in the procedure at pilot scale.

3.3. Clarification of β -D-glucan-rich extracts by cross-flow microfiltration

3.3.1. Effect of the physicochemical characteristics of the extracts on the main parameters of operation

Fine clarification of the cold-settled Shiitake extracts was carried out by cross-flow microfiltration, at the low-pressure mode (P_{TM} 0.7 bar, 20 °C). The kinetics analysis of the permeate fluxes of the three studied Shiitake extracts (E1, E2 and E3) (Fig. 4) indicated that the highest filtration flux was recorded during extract clarification with 2.9% TSS (E3) (Table 3, Fig. 5c). In this case, 80 L extract were concentrated to 7 L (F_C of 11.4) and 73 L filtrate was recovered, without breaks or any operational problem in < 20 h. The treatment started at 77 L/hm² and diminished down to 49 L/hm² at the end indicating a really good filtration flux. It could also be noticed that the extract flux with the highest TSS content (4.2%) (E2) was much lower (Table 3, Fig. 5b), comprising the interval of 43–20 L/hm². In this case, 55 L were concentrated to 7 L (F_C of 7.9) for 14 h and 48 L of filtrate was recovered. The problem with this extract was not only the lowest filtration flux, but also some difficulties related to partial obstruction of the equipment tubing with insoluble particles and subsequent breaks of the process for purging the equipment tubing to reestablish filtration. Extract E1 with the lowest TSS content (1.9%) was filtered without operational problems, but showed lower filtration flux (65–47 L/hm²) than E3, effect which could be explained with the higher TSP (turbidity) load (Table 3). As filtration was carried out by concentration of an initial volume of 20 L of each extract, an effect of concentrate dilution was produced at each addition of 5 L of unfiltered extract (Fig. 5),

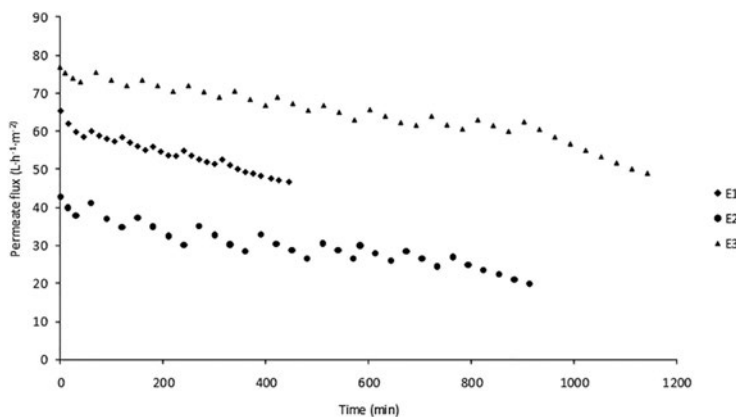


Fig. 4. Permeate flux kinetics ($\text{Lh}^{-1}\text{m}^{-2}$) during cross-flow microfiltration of the studied Shiitake extracts, E1, E2 and E3.

resulting in certain increase of the filtration fluxes (Fig. 4). In all cases, filtration fluxes were inversely proportional to the increase of the TSS contents of the extracts (Fig. 5) and to the increase of the total suspended particle concentration (turbidity) in the concentrate streams (Fig. 6). The first effect was more evident between extracts than during filtration of each of them (Fig. 5), whereas the second was most easily appreciable during filtration than between extracts (Fig. 6).

The observed permeate flux kinetics of the studied extracts showed faster flux declines at the beginning of the treatments and establishment of steady fluxes afterwards. This effect is usually related to the buildup of a secondary fine-particle/colloidal layer on the membrane surface and depends mostly of the TSP and the colloidal part of the TSS.

When TSS content of the extracts was relatively low (1.9–2.9% for

E1 and E3, respectively), the transference of TSS throughout the membrane was high (between 90 and 75%), but not entirely, leading to retention of some soluble compounds in the part of the concentrate (Fig. 5). In the case of the extract with the highest TSS load (4.2%) (E2) these values were considerably lower, 60–55%. These findings indicate that the filtration flux is inversely related to the TSS load and suggest that, although the $0.5\mu\text{m}$ pore size membrane was rated as a micro-filtration membrane, it rather acted as an ultrafiltration membrane.

The concentrates obtained after the three microfiltration treatments contained the finest fraction of insoluble β -D-glucans. They could be added to the insoluble fraction left after the extraction of the soluble β -D-glucans, but they were discarded because of their high microbial load.

With respect to the TSP load of the extracts, the turbidities of the

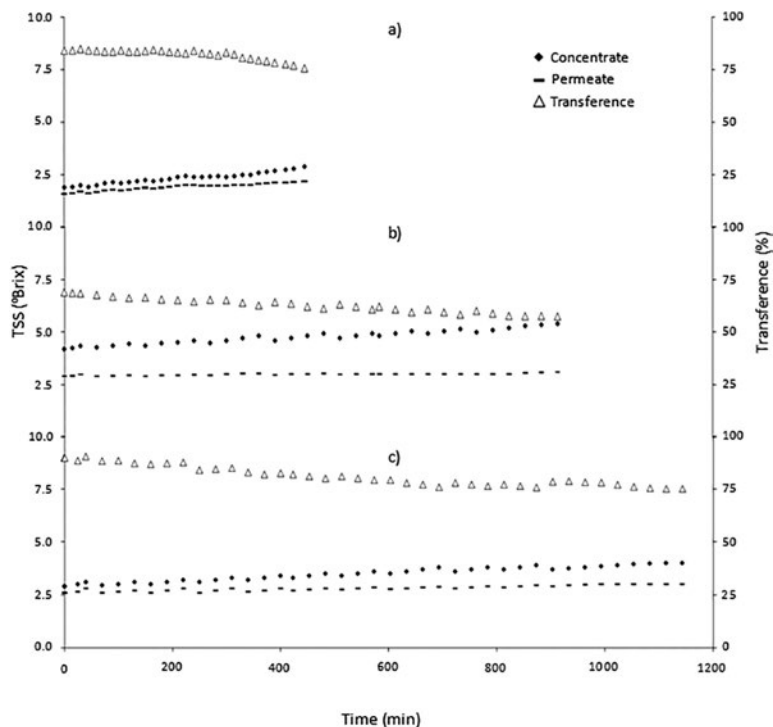


Fig. 5. Distribution of total soluble substances (TSS) in the concentrate and permeate flows and transference of TSS throughout the membrane during cross-flow microfiltration of the studied Shiitake extracts E1 (a), E2 (b) and E3 (c). (\diamond -TSS_{conc}, total soluble substances in the concentrate; TSS_{perm}, total soluble substances in the permeate; Δ -TSS_{transf}, transference of total soluble substances through the corresponding membrane.)

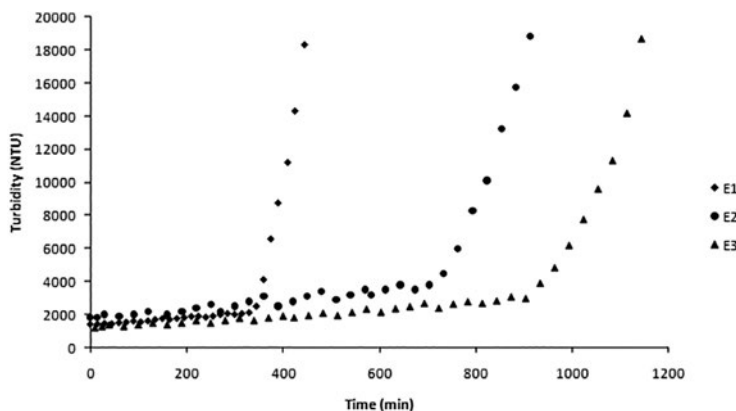


Fig. 6. Kinetics of turbidity (NTU) in the concentrate flow during microfiltration of the studied Shiitake extracts E1, E2 and E3.

three cold-settled extracts were very high (Table 3), comprising the interval of 1213 (E3) and 1832 NTU (E2). The operational problems appeared during filtration of the E2 suggested that turbidities higher than 1800 were inappropriate for this process and should be avoided.

As filtrations were carried out by addition of 5 L fractions of unfiltered extracts (to the 15 L concentrates), only small increases of turbidity were observed during these periods (Fig. 6), while the concentration of the last 15 L of each extract resulted in an exponential turbidity increase. That is why the filtration of the 45 L of E1 entered in this exponential phase earlier than the 55 and 80 L of respectively E2 and E3.

The results from Figs. 4–6 suggest that for an optimal cross-flow microfiltration, TSS of the Shiitake extracts should be around 3%, but not higher than 3.5% (substrate to solvent ratio around 60 g/L or lower than 65 g/L) and that it was possible to clarify volumes higher than 80 L/0.1 m² (800 L/m²) of membrane filtration surface in one concentration batch (without discharge of the concentrate). But probably, the most important feature of these experiments was the complete recovery (100%) of the membrane permeability after standard regeneration with 0.5 M NaOH solution, which revealed the high physical and chemical resistance of this membrane in the clarification of Shiitake extracts with high loads of suspended particles.

3.3.2. Effect of the cross-flow microfiltration on the quality of the soluble β -glucan-rich extracts and recovery of the rests of insoluble β -D-glucans

The effect of the 0.5 μ m pore size membrane on the clarification of the three studied Shiitake extracts (E1, E2 and E3) was excellent, giving turbidities in the narrow interval of 0.3–0.6 NTU (Table 3). These low turbidities values indicated the complete separation of the soluble from the insoluble β -D-glucans and warranted the microbiological stabilization of the obtained soluble β -glucan-rich extracts. In fact, the filtrates were microbiologically stable during several weeks whereas the concentrates developed off-odors in few hours after treatment if they were not frozen.

Nevertheless, the data from Fig. 5 also showed that this membrane retained up to 25% of TSS in all of the studied extracts and this retention was directly proportional to the increase of the TSS concentration in the concentrate flow. These results indicated that some parts of the high molecular mass compounds (most probably soluble β -D-glucans) were retained in the suspended particle fraction. Partial recovery of these compounds is possible by the introduction of an additional diafiltration step of the final concentrate or by decreasing the volume concentration factor (F_C) of the process. The first option would lead to an increase of operation time and expenses for elimination of the added water and the second one would decrease the volume of the filtered extract. However, results from the analysis of β -D-glucan

content of the studied extracts (Fig. 10) showed that the retention of β -D-glucan in the concentrates was insignificant in the three cases.

3.4. Concentration of clarified β -glucan-rich extracts by reverse osmosis (RO)/nanofiltration (NF)

3.4.1. Effect of the physicochemical characteristics of the extracts on the operation parameters

For concentration of the clarified Shiitake extracts one RO and two NF membranes were tested, using the pressure-driven membrane unit described in Fig. 1, set at the high-pressure mode. Thus, 38 L of the clarified E1 extract with 2.2% of TSS were concentrated to 6 L with 7.2% of TSS (Fig. 8a) ($F_C = 6.3$) by the polyamide RO (Nanamax95) membrane at P_{TM} of 24 bar (20 °C) for 4.3 h, without any operational problem. During the treatment, the filtration flux dropped from 30 to 18 L/hm² in an almost lineal mode (Fig. 7), which denotes 40% loss of the initial flux.

Then, 48 L of the clarified E2 extract with 3.1% of TSS were concentrated to 6 L with 15.4% of TSS (Fig. 8b) ($F_C = 8$) by the polyamide NF (Nanamax50) membrane (0.37 m² of filtration surface) at P_{TM} of 29 bar (20 °C). In this case, a membrane with 65% NaCl rejection (96% MgSO₄ rejection, Table 1) was used and an initial filtration flux of 119 L/hm² was achieved, even the higher TSS load of this extract (Fig. 8b). Nevertheless, this flux dropped to 38 L/hm² in 1.5 h which means almost 68% loss of the initial flux.

Finally, 74 L of the clarified E3 extract with 3.0% of TSS were concentrated to 7 L with 13.6% of TSS (Fig. 8c) ($F_C = 10.6$) with a NF (ALNF99) membrane, at P_{TM} of 26 bar (20 °C). This membrane had a larger filtration surface (1 m²) and > 98% rejection of MgSO₄ (Table 1), but an initial filtration flux of 75 L/hm² was achieved, even the TSS load of this extract was lower than the E2 (Fig. 8). In this case the loss of filtration flux was smaller (around 65%), which is also due to the lower TSS concentration of the extract.

As it was expected, the obtained results showed that the Nanamax95 reverse osmosis membrane had 2.5 to 4 times lower initial filtration flux than the two nanofiltration membranes, ALNF99 and Nanamax50, respectively, even that the E1 extract had the lowest TSS load. This can be clearly related to the high rejection rate of this membrane and the slightly lower pressure at which the treatment was carried out. The difference found between the initial fluxes of the two nanofiltration membranes were not negligible and should be related to differences in the nature of the materials, as Nanamax50 was a polyamide membrane and ALNF99 was not specified (but not polyamide). In this context, it should be highlighted that the ALNF99 membrane lost completely its retention capacity after the next 2 months of storage at the conditions specified by the producer. No clear reasons for this

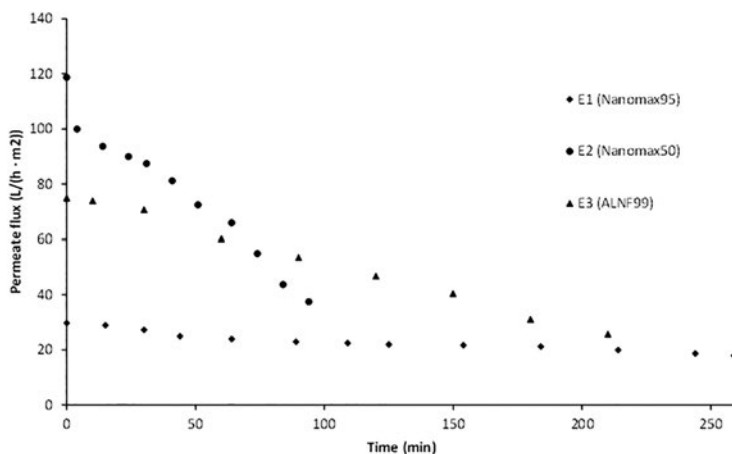


Fig. 7. Permeate flux kinetics ($\text{Lh}^{-1} \text{m}^{-2}$) during concentration of the clarified E1, E2 and E3 Shiitake extracts by the corresponding to each of them membranes Nanomax95, Nanomax50 and ALNF99.

failure were found and the use of this membrane was abandoned.

Moreover, in all cases, filtration fluxes decreased in linear modes depending directly of the TSS concentration in the concentrate streams (Figs. 7 and 8) and this dependence was more pronounced as higher was the initial TSS load of the extracts. On the other hand, there was no zone of steady flux, as it was observed in the case of the clarification treatments (Fig. 4), mostly due to the absence of suspended particles and/or colloids in the extracts indicating that there was no evidence for formation of concentration-polarization phenomena in these treatments.

The obtained results showed that the treatments with the studied RO and NF membranes allowed reducing the initial volumes of the fine-clarified extracts to volumes of 6–7 L, completely suitable for direct drying.

3.4.2. Effect of the concentration on the content of soluble β -D-glucans

For evaluation of the effect of concentration of the studied Shiitake extracts a criteria of minimal losses of soluble β -D-glucans and higher removal of other substances was adopted. For this, electrical conductivity was measured during the concentration process. Solution electrical conductivity is basically related to the content of inorganic and organic electrolytes, such as mineral salts, carboxylic acids, some amino acids and peptides, among others. Non-electrolytes, such as carbohydrates have zero or very low electrical conductivity, thus measurement of this parameter during concentration of carbohydrate solutions may be very indicative for separation of the electrolytes. In addition, it is an easy and fast measurement which can be carried out during the treatment.

The results for electrical conductivity, measured during concentration of the clarified E1, E2 and E3 Shiitake extracts (Fig. 9) showed that the RO membrane Nanomax95 retained almost completely all electrolytes in the concentrate, i.e. there was no separation from the carbohydrates. The treatment with the NF membrane ALNF99 produced certain transference of electrolytes (up to 5%), mostly at the last concentration period, whereas the Nanomax50 NF membrane showed the highest transfer of electrolytes, with values comprised in the interval of 32 to 25% at the end of concentration. These results indicate that the Nanomax50 membrane not only concentrated the Shiitake β -D-glucans, but also purified them from an important part of the extract electrolytes.

3.5. Evaluation of the global processing of β -glucan-rich extracts at molecular level

β -D-Glucan contents were quantified in the initial cold-settled Shiitake extracts (E1, E2 and E3), their corresponding fine clarified extracts (microfiltered permeates, MP1, MP2 and MP3) and concentrates obtained by Nanomax95 (RF1), Nanomax50 (RF2) and ALNF99 (RF3) membranes (Fig. 10). Results indicated that, in general, the MF process did not produce any change of β -D-glucan concentration of the most concentrated E2 and E3 extracts (2.9 and 4.2% of TSS), whereas a small but significant loss of 20% was observed in the extract with the lower concentration of TSS (1.9%, E1) (Fig. 10). With respect to the concentration treatment, the RO Nanomax95 and the NF ALNF99 membranes had no effect on the β -D-glucan contents of the extracts, whereas the NF Nanomax50 (RF2) produced a low, but significant β -D-glucans enrichment (21%), due basically to the removal of some electrolytes. Having in mind the highest filtration flux obtained by this membrane, it could be suggested that Nanomax50 was the most adequate membrane for this treatment.

Beside β -D-glucans, other bioactive compounds were determined in the obtained extracts, since they also exhibited cardiovascular protective effects such as eritadenine, ergosterol, lenthionine - an organo-sulfur compound which showed capacity to inhibit platelet aggregation in vitro (Shimada, Komamura, Kumagai, & Sakurai, 2004) - and chitins as source of chitosan derivatives.

The aqueous extracts E1, E2 and E3 contained between 28 and 35% (w/w) of total carbohydrates (TC) (Table 4), amounts relatively low compared to those of the insoluble residues (R1, R2, R3) (48–53%) indicating that relevant amounts of mushroom carbohydrates remained in the solid phase, which could not be extracted with boiling water. This result was already observed particularly for the β -D-glucans (Fig. 2) and is extensible for other polysaccharides, such as chitins (Table 4). In general, the MF/NF/RO treatments did not affect significantly the total carbohydrate and chitin contents all over the treatment. The highest concentration ratio of total carbohydrates (TC after concentration/TC before concentration) was achieved when Nanomax50 NF membrane was used (1.09), followed by the ALNF99 NF (1.03) and Nanomax95 RO membrane (0.89). Significantly lower amounts of chitins were found in the extracts before and after MF/NF/RO treatments, indicating that only a remaining fraction of low molecular mass and degradation derivatives from chitins were present after filtration. These chitin products were also observed in previous reports (Palanisamy et al., 2014).

Results also indicated that eritadenine was easily extracted with

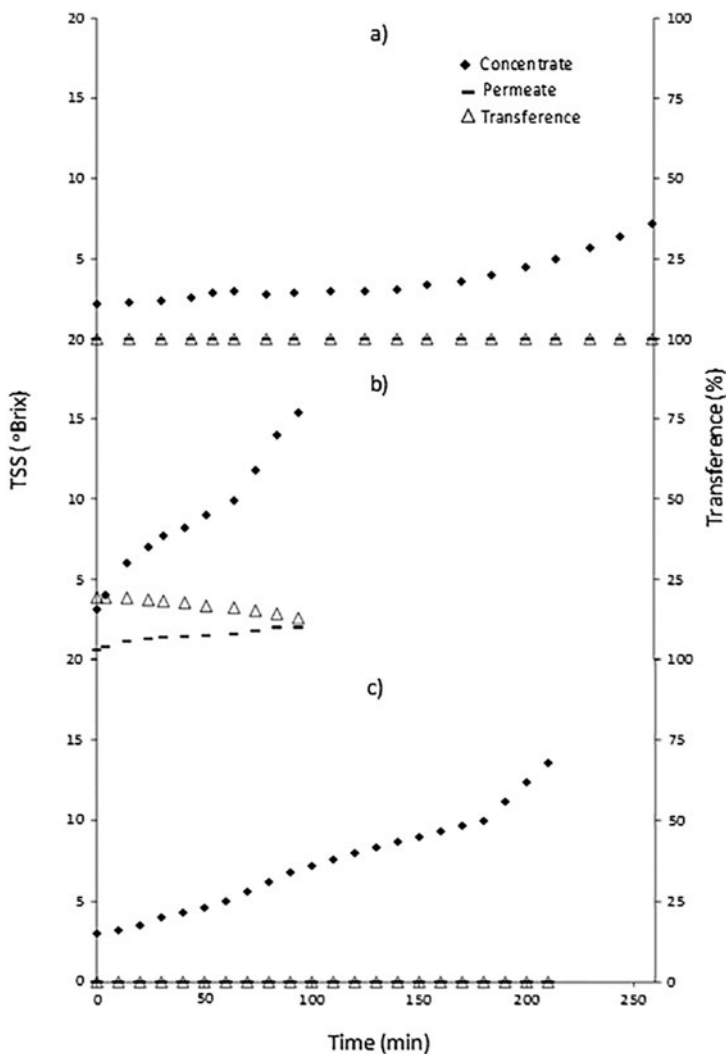


Fig. 8. Kinetics of the total soluble substance contents (TSS) during concentration of the clarified E1, E2 and E3 Shiitake extracts by the corresponding to each of them membranes Nanomax95 (a), Nanomax50 (b) and ALNF99 (c) (\diamond -TSS_{concs}, total soluble substances in the concentrate; TSS_{perm}, total soluble substances in the permeate; Δ -TSS_{transf}, transference of total soluble substances through the corresponding membrane.)

boiling water and that the MF/NF/RO treatments increased its concentration up to 6.9 mg/g, indicating that this water soluble compound resists high temperatures as previously reported. On the contrary, lenthionine that was present in the Shiitake powder before heating was not detected in any of the obtained extracts confirming the results of Shiga et al., 2014 indicating that lenthionine suffers thermal degradation above 80 °C.

Ergosterol, a lipophilic compound with hypocholesterolemic effect was mainly found in the insoluble residue at concentrations approx. 5 mg/g (Table 4). These values were higher than their usual levels in mushroom hyphae, therefore a 2.1 fold concentration ratio was obtained and the insoluble fraction might be considered for compound recovery.

These results pointed out that, besides the soluble β -D-glucans, other interesting carbohydrates and eritadenine can be extracted with boiling water from Shiitake mushrooms. However, the insoluble fractions could

also be utilized as a source of hypocholesterolemic compounds such as insoluble β -D-glucans, chitins and ergosterol.

4. Conclusions

Maceration of Shiitake powder in water at 98 °C for 1 h at pilot scale (30 L) led to the production of up to 80 L of β -glucan-rich extracts with concentrations of 1.9–4.2% total soluble substances (TSS). Higher or longer heat exposure of the substrate (> 2 h at 98 °C and only 20 min at 120 °C) had negative effect on β -D-glucan content. The optimal substrate to solvent ratio was established as 50 g/L, being higher ratios detrimental for the following microfiltration process.

> 80 L of Shiitake extracts with up to 2.9% TSS and up to 1400 NTU turbidity could be successfully filtered by the studied CeraMem microfiltration membrane (0.5 μ m pore size and 0.1 m² filtration surface). The treatment produced an outstanding extract

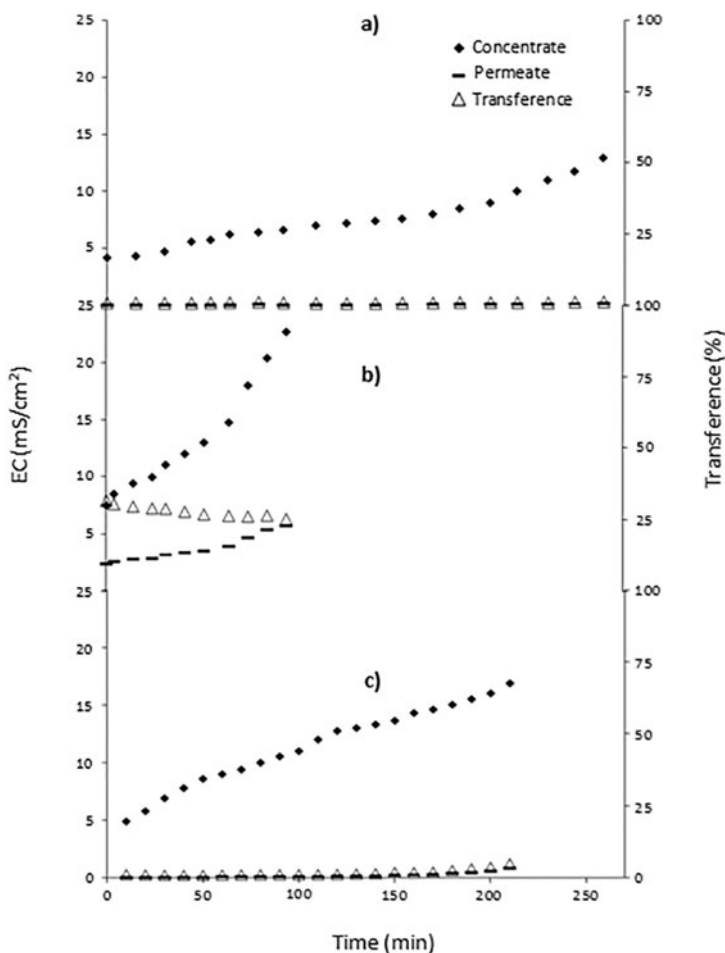


Fig. 9. Electrical conductivity (EC) during concentration of the clarified E1, E2 and E3 Shiitake extracts with Nanomax95 (a), Nanomax50 (b) and ALNF99 (c), membranes, respectively. (\diamond -TSS_{conc}, total soluble substances in the concentrate; TSS_{perm}, total soluble substances in the permeate; Δ -TSS_{transf}, transference of total soluble substances through the corresponding membrane.)

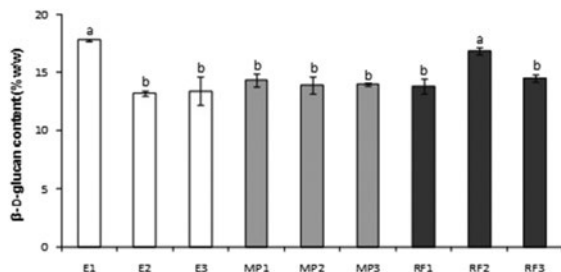


Fig. 10. β -D-Glucan contents (% w/w) of the three cold-settled (E1, E2 and E3), fine clarified (MP1, MP2 and MP3) and concentrated Shiitake extracts by Nanomax95 (RF1), Nanomax50 (RF2) and ALNF99 (RF3) membranes. Different letters (a–b) denote significant differences ($P < 0.05$) between values of the same series.

clarification, reducing turbidity to < 0.5 NTU. Filtration fluxes of $70\text{--}60\text{ L/hm}^2$ could be easily obtained at relatively low transmembrane pressure (0.7 bar) and temperature (20°C). Worth to mention was the

fact that membrane permeability was completely recovered after filtration of so heavy suspensions. Nevertheless, it should be indicated that extracts with higher suspended particle loads might be detrimental for the membrane performance and should be avoided. Cold-settling contributed positively to the successful extracts clarification.

Large volumes ($> 70\text{ L}$) of clarified extracts with $2.2\text{--}3.1\%$ TSS could be efficiently concentrated to $6\text{--}7\text{ L}$ by reverse osmosis or nano-filtration, corresponding to concentration factors of 8 to 10. Filtration fluxes greatly depend of the content of extract TSS and their decrease was faster when the initial content of the extract TSS was higher. Values of $80\text{ to }20\text{ L/hm}^2$ were usual at transmembrane pressure of $25\text{--}29\text{ bar}$ and temperature of 20°C . The Nanomax50 NF membrane gave higher filtration flux.

Independently of the variations tested during the extraction and filtration treatments, extracts with $13.8\text{--}16.9\%$ (dry weight) β -D-glucans were obtained. However, effective enrichment of β -D-glucans was achieved only in the treatment carried out with the Nanomax50 NF membrane, due to its higher capacity to eliminate electrolytes. Apart from β -glucans, the obtained extracts contained also other hypocholesterolemic molecules such as eritadenine at concentration of up to 7 mg/g .

Table 4

Concentration of bioactive compounds (total carbohydrates (TC), chitins (CH), eritadenine (EA), lenthionine (LT) and ergosterol (ER)), measured in Shiitake powder (SP), the water-insoluble fractions (R1, R2, R3) and the cold-settled (E1, E2 and E3), fine clarified (MP1, MP2, MP3) and concentrated by Nanomax95 (RF1), Nanomax50 (RF2) and ALNF99 (RF3) membranes Shiitake extracts. Different letters (a–c) denote significant differences ($P < 0.05$) between values of the same series.

	TC	CH	ER	EA	LT
	(% w/w)	(% w/w)	(mg/g)	(mg/g)	(mg/g)
SP	40.67 ± 0.65 ^b	6.03 ± 0.29 ^b	2.40 ± 0.02 ^b	1.44 ± 0.14 ^c	0.15 ± 0.01 ^a
R1	49.03 ± 4.18 ^a	9.08 ± 0.87 ^a	4.81 ± 0.05 ^a	0.57 ± 0.02 ^c	n.d. ^b
R2	48.01 ± 3.09 ^{ab}	9.12 ± 0.46 ^a	5.00 ± 0.48 ^a	1.01 ± 0.03 ^c	n.d. ^b
R3	53.36 ± 1.32 ^a	9.25 ± 0.76 ^a	4.69 ± 0.21 ^a	0.31 ± 0.03 ^c	n.d. ^b
E1	35.06 ± 3.75 ^b	1.85 ± 0.97 ^c	0.75 ± 0.04 ^c	4.49 ± 0.69 ^b	n.d. ^b
E2	28.14 ± 0.86 ^c	2.04 ± 0.37 ^c	0.26 ± 0.01 ^{cd}	4.37 ± 0.51 ^b	n.d. ^b
E3	30.53 ± 1.79 ^c	1.53 ± 0.42 ^c	0.73 ± 0.02 ^c	5.41 ± 0.11 ^{ab}	n.d. ^b
MP1	34.69 ± 0.74 ^b	1.30 ± 0.28 ^c	n.d. ^d	6.00 ± 0.02 ^{ab}	n.d. ^b
MP2	27.99 ± 1.05 ^c	1.91 ± 0.12 ^c	n.d. ^d	5.46 ± 0.21 ^{ab}	n.d. ^b
MP3	29.83 ± 0.38 ^c	0.69 ± 0.05 ^c	n.d. ^d	6.11 ± 0.26 ^{ab}	n.d. ^b
RF1	30.74 ± 1.73 ^c	2.38 ± 0.56 ^c	n.d. ^d	6.93 ± 1.29 ^a	n.d. ^b
RF2	30.63 ± 2.00 ^c	2.12 ± 0.05 ^c	n.d. ^d	5.72 ± 0.02 ^{ab}	n.d. ^b
RF3	30.70 ± 1.13 ^c	1.06 ± 0.06 ^c	n.d. ^d	6.89 ± 0.31 ^a	n.d. ^b

Nevertheless, it must be emphasized that 36–41% of water insoluble β-D-glucans and other interesting compounds such as ergosterol and chitins (respectively 0.5 and 9% (w/w)), remained in the insoluble fraction, suggesting that this fraction might be an important source of these compounds.

Conflict of interest

None.

Acknowledgments

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Isolation and comparison of α - and β -D-glucans from shiitake mushrooms (*Lentinula edodes*) with different biological activities

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ABSTRACT

A polysaccharide-enriched extract obtained from *Lentinula edodes* was submitted to several purification steps to separate three different D-glucans with β -(1 \rightarrow 6), β -(1 \rightarrow 3), (1 \rightarrow 6) and α -(1 \rightarrow 3) linkages, being characterized through GC-MS, FT-IR, NMR, SEC and colorimetric/fluorimetric determinations. Moreover, *in vitro* hypocholesterolemic, antitumoral, anti-inflammatory and antioxidant activities were also tested. Isolated glucans exerted HMGR inhibitory activity, but only β -(1 \rightarrow 6) and β -(1 \rightarrow 3), (1 \rightarrow 6) fractions showed DPPH scavenging capacity. Glucans were also able to lower IL-1 β and IL-6 secretion by LPS-activated THP-1/M cells and showed cytotoxic effect on a breast cancer cell line that was not observed on normal breast cells. These *in vitro* results pointed important directions for further *in vivo* studies, showing different effects of each chemical structure of the isolated glucans from shiitake mushrooms.

1. Introduction

Mushroom D-glucans showed interesting industrial applications in agronomic, food, cosmetic and therapeutic areas. Such glucans might present different branching degrees, molecular mass and solubility (Borchani et al., 2016; de Jesus et al., 2018). Therefore, the correlations between their chemical structures and their biological properties were deeply studied. According to their anomericity, it is possible to encounter α -D-glucans and β -D-glucans in mushroom fruiting bodies, although mixed α / β -D-glucans were also described (Synytsya & Novak, 2013). The anomericity associated with different linkages may drastically influence tridimensional configuration and solubility; consequently, it might also modulate glucan bioactivities (Benito-Roman, Martín-Cortes, Cocero, & Alonso, 2016; Zhang, Cui, Cheung, & Wang, 2007).

The most commonly isolated glucans from fungi are β -D-glucans, and a large variety of beneficial effects on human health was described for them, such as immunomodulatory, antitumoral, hypolipidemic or

antimicrobial activities (Khan, Gani, Khanday, & Massodi, 2018). However, α -D-glucans and mixed α / β -D-glucans were less frequently isolated although they both showed antioxidant activities (Maity et al., 2017). Furthermore, α -D-glucans were also described as compounds with interesting immunomodulatory, antitumoral, hypoglycemic and hypolipidemic properties (Hong, Weiye, Qin, Shuzhen, & Iebin, 2013; Lei et al., 2013; Masuda, Nakayama, Tanaka, Naito, & Konishi, 2017).

Shiitake (*Lentinula edodes*) is the most popular edible mushroom in global market (Royse, Baars, & Tan, 2017), highly valued in oriental and recently in occidental cuisine because of their characteristic flavor. This mushroom includes molecules inducing positive effects on human health such as phenolic compounds and ergothioneine (antioxidant activity), ergosterol, β -glucans, and eritadenine (hypocholesterolemic properties), antihypertensive peptides, lenthionine (with antithrombotic capacity), among others (Morales, Piris, Ruiz-Rodríguez, Prodanov, & Soler-Rivas, 2018), however, lentinan deserves special attention. It is a well-characterized glucan consisting of a main chain of (1 \rightarrow 3)-linked β -D-glucopyranose units, substituted at O-6 by β -D-

Abbreviations: G-1, Glucan-I; G-2, Glucan-II; G-3, Glucan-III; HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase

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glucopyranose, at a frequency of two branches for every five units from the main chain. This polysaccharide attracted clinical interest because of its strong *in vitro* and *in vivo* antitumor action as well as immunomodulatory and antiviral capacities (Zhang, Li, Wang, Zhang, & Cheung, 2011). Moreover, certain α -D-glucans such as an (1 \rightarrow 3)- α -D-glucan and glycogen ((1 \rightarrow 4),(1 \rightarrow 6)- α -D-glucan) were also detected in MAE (microwave-assisted extraction) and hot water extractions (Gil-Ramirez, Smiderle, Morales, Iacomini, & Soler-Rivas, 2019; Morales, Smiderle, Villalva et al., 2019), although their potential bioactivities are nowadays not so well studied as lentinan.

Several purification procedures were developed to separate these molecules and to test their individual bioactivities. Freeze-thawing separation, treatment with solvents, dialysis, ultrafiltration and column fractionation were usually utilized since they are simple methods (Ruthes, Smiderle, & Iacomini, 2015), however, polysaccharides frequently form intermolecular interactions yielding complex polymers difficult to isolate. Mushroom glucans also showed this tendency but a recent study indicated a simple and effective procedure to solve this issue and separate different glucan structures (de Jesus et al., 2018).

In this work, a crude polysaccharide fraction obtained from shiitake mushrooms was submitted to the novel procedure and three different glucans were isolated: a branched (1 \rightarrow 3),(1 \rightarrow 6)- β -D-glucan, a linear (1 \rightarrow 3)- α -D-glucan and a mixed fraction composed mainly by a linear (1 \rightarrow 6)- β -D-glucan with low levels of (1 \rightarrow 3)- β -D-glucan. The chemical structures were defined by colorimetric/fluorimetric procedures, GC-MS, SEC, FT-IR and NMR. Furthermore, the antioxidant and hypcholesterolemic activities of the glucans were tested *in vitro* and their immunomodulatory effects and antitumor properties were investigated using cell cultures on THP-1 and breast tumor cell lines, respectively.

2. Experimental

2.1. Fungal material

Powdered *Lentinula edodes* S. (Berkeley) fruiting bodies (particle size < 0.5 mm, moisture < 5%) were purchased from Glucanfeed S.L. (La Rioja, Spain) and stored in darkness at -20°C until further use.

2.2. Reagents

Absolute ethanol was obtained from Panreac and sodium borohydride (NaBH_4), sodium hydroxide pellets, glycine, D-glucose, glucosamine hydrochloride, aniline blue diammonium salt 95%, trifluoroacetic acid, pyridine, acetic anhydride, copper(II) sulfate (CuSO_4), deuterated dimethylsulfoxide ($\text{Me}_2\text{SO}-d_6$), Congo Red, citric acid, dextran (M_w 35,000–45,000 g/mol), RPMI 1640 medium and phorbol 12-myristate 13-acetate (PMA), DPPH (2,2-diphenyl-1-picrylhydrazyl), DMEM medium, dimethyl-sulfoxide, ascorbic acid, horse serum, fetal bovine serum, hydrocortisone, recombinant EGF and insulin were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA).

2.3. Extraction and purification of polysaccharides

Shiitake powder was submitted to hot water extraction (98°C , 1 h) as described by Morales, Smiderle, Piris, Soler-Rivas, and Prodanov (2019) and the soluble fraction was previously described by these authors. The insoluble fraction containing high levels of glucans (40% β -D-glucans dry weight) was utilized to carry out the purification procedures (Fig. 1). Ethanol precipitation was performed by adding 3 volumes of ethanol, mixing vigorously and keeping the mixture overnight at 4°C . The precipitated polysaccharides were recovered after centrifugation (10,000 rpm, 15 min) and the pellets were suspended in water and dialysed (2 kDa M_r cut-off membrane) against water for 24 h. The crude polysaccharides were freeze-dried and submitted to the first alkaline treatment (stirring with 0.01 M NaOH solution at 22°C , for 1 h). After this period, the solution was cooled down to 4°C and then

centrifuged (8000 rpm, 10°C , 20 min). Soluble (S-1) and insoluble (I-1) polysaccharides resulting from the alkaline treatment were neutralized with acetic acid and dialysed (2 kDa M_r cut-off membrane) against water for 24 h and then freeze-dried. S-1 was submitted to a freeze-thawing process (Gorin & Iacomini, 1984), and subdivided into two new fractions based on their solubility in water: Glucan-I (G-1) and Glucan-II (G-2). Due to high insolubility, fraction I-1 was submitted to a second and stronger alkaline treatment (stirring with 0.1 M NaOH solution; at 22°C , for 1 h) (de Jesus et al., 2018), yielding two new fractions, although only the insoluble one (named Glucan-III, G-3) was used in this study. Extraction yields were calculated based on the initial dry weight of shiitake mushroom powder.

2.4. GC-MS analysis

The monosaccharide composition of the fractions (G-1, G-2, and G-3) was determined by hydrolyzing the samples (1 mg) with 2 M trifluoroacetic acid at 100°C for 8 h followed by evaporation to dryness. The dried samples were dissolved in distilled water (100 μL) and NaBH_4 (1 mg) was added. Then, solution was kept at room temperature overnight to reduce aldose into alditols (Sasaki et al., 2008) and later, the samples were dried and the NaBH_4 excess was neutralized by adding acetic acid and then removed with methanol (twice) under a compressed air stream. Alditols acetylation was performed in pyridine-acetic anhydride (200 μL ; 1:1 v/v) for 30 min at 100°C . Pyridine was removed by washing with 5% CuSO_4 solution and the resulting alditol acetates were extracted with chloroform. The samples were injected into an SH-Rtx-5 ms (30 m \times 0.25 mm ID \times 0.25 μm thickness phase). The column was connected to a GC-2010 Plus gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a Combipal autosampler (AOC 5000) and coupled to a triple quadrupole mass spectrometer TQ 8040. The injector and ion source were held at 250°C and helium at 1 mL/min was used as carrier gas. The oven temperature was programmed from 100 to 280°C at $10^{\circ}\text{C}/\text{min}$ with a total analysis time of 30 min. The samples were prepared in hexane with 1 μL being injected with a split ratio of 1:10. The mass spectrometer was operated in the full-scan mode over a mass range of m/z 50–500 before selective ion monitoring mode, both with electron ionization at 70 eV. Selective ion monitoring mode was used for quantification and GCMS solution software (Tokyo, Japan) was used for data analysis. The obtained monosaccharides were identified by their typical retention time compared to commercial available standards. Results were expressed as mol%, calculated according to Pettolino, Walsh, Fincher, and Bacic (2012).

2.5. NMR spectroscopy

NMR spectra (^1H , ^{13}C and HSQC-DEPT) from the different fractions were obtained using a 400 MHz Bruker model Advance III spectrometer with a 5 mm inverse probe, and the analyses were performed at 70°C . The samples (30 mg) were dissolved in $\text{Me}_2\text{SO}-d_6$ and were centrifuged (10,000 rpm, 22°C , 2 min) to remove insoluble material, therefore only the soluble fractions of G-1, G-2 and G-3 were analyzed. Chemical shifts are expressed in ppm (δ) relative to $\text{Me}_2\text{SO}-d_6$ at 39.7 (^{13}C) and 2.40 (^1H).

2.6. FT-IR and SEC analyses

Infrared analysis was performed in a Vertex 70 spectrometer (Bruker, Germany) with attenuated total reflectance (ATR). Aliquots of the dried samples G-1, G-2, and G-3 were prepared using KBr disc technique and directly submitted to infrared analysis with 32 scans from 410 to 4000 cm^{-1} with resolution of 4 cm^{-1} .

SEC analysis was performed at 40°C using as mobile phase NaNO_3 0.1 mol/L containing sodium azide 200 ppm under a flow rate of 0.4 mL/min in a Viscotek-SEC multidetector-system. This system was equipped with an OH-Pack Shodex SB-806 M HQ column (size

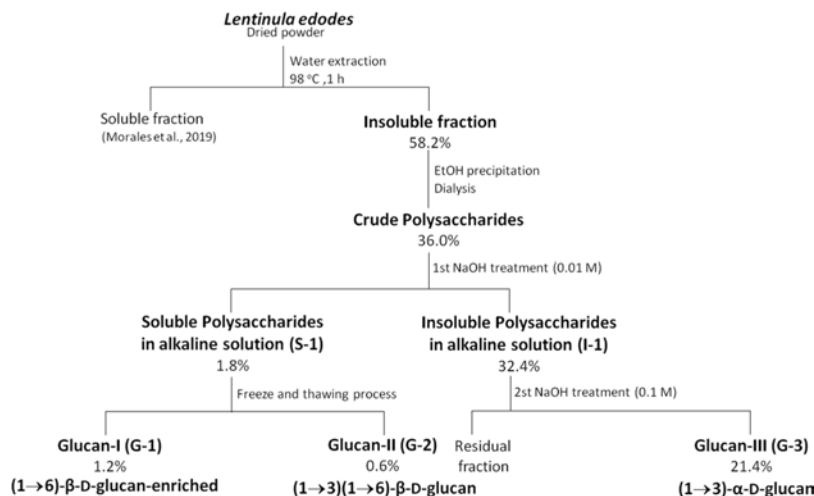


Fig. 1. Scheme of extraction and purification of glucans obtained from shiitake powder. Indicated yield (%) was calculated on basis of the initial dry weight of shiitake mushroom powder.

exclusion limits of 2×10^7 g/mol) coupled to laser light scattering detector model 270 dual detector with low angle 7° (LALLS) and right angle 90° (RALLS) with λ at 632.8 nm and to a RI (Viscotek VE3580) detector. Aliquots of samples were dissolved in the eluent (1 mg/mL) and then filtered through 0.22 μ m cellulose membrane prior to injection. Results were analyzed with OmniSEC software (Malvern Co., USA) and Mw was calculated only for soluble samples.

2.7. Colorimetric determinations with Congo red

Determination of triple helix conformation was performed according to Smiderle et al. (2014). Congo red was dissolved (80 μ M) in 50 mM NaOH solution. Dextran (1 mg/mL) was used as random coil control and Congo red alone was considered as negative control. Studied samples (G-1, G-2, G-3) were added (1 mg/mL) to Congo red solutions and spectra were recorded on an Evolution 600 UV–vis spectrophotometer (ThermoFisher Scientific, Spain) in intervals of 10 nm from 400 to 640 nm.

2.8. Fluorimetric determinations

The determination of (1→3)- β -D-glucans was carried out according to Gil-Ramirez et al. (2019). Briefly, purified samples (G-1, G-2, G-3) were solubilized (2.5–100 μ g/mL) in 300 μ L of 0.05 M NaOH with 1% NaBH₄ in 2 mL reaction tubes. After that, 30 μ L of 6 M NaOH and 630 μ L of dye mix (0.1% aniline blue: 1 M HCl: 1 M glycine / NaOH buffer pH 9.5; 33:18:49) was added and the mixed samples were incubated at 50 °C for 30 min in a water bath and transferred to a 96-well plate to carry out fluorimetric analysis (excitation: 398 nm; emission: 502 nm) in a M200 Plate Reader (Tecan, Mannedorf, Switzerland).

2.9. Determination of HMGCR inhibitory activity

Purified samples were solubilized in water (G-1) or water/DMSO (G-2, G-3, 1:0.063, 10 mg/mL) and applied (20 μ L) into a 96-wells plate. Their inhibitory activity was measured using the commercial HMGCR (3-hydroxy-3-methylglutaryl coenzyme A reductase) activity assay (Sigma-Aldrich, Madrid, Spain) according to the manufacturer's instructions by monitoring their absorbance change (340 nm) at 37 °C using a 96-wells microplate reader BioTek Sinergy HT (BioTek, Winooski, USA). Pravastatin was used as a control for positive

inhibition.

2.10. Determination of free radical scavenging activity

The scavenging activity of the isolated glucans against the stable free radical DPPH[•] (2,2-diphenyl-1-picrylhydrazyl) was determined, using different concentrations of the fractions G-1, G-2 and G-3 (1000; 300; 100; 30; 10; 3; and 1 μ g/mL). This method was adapted from Kanazawa et al. (2016). Briefly, the tested fractions were, separately, mixed with DPPH methanol solution (40 μ g/mL), and absorbance was immediately read at 517 nm in an Epoch Microplate Spectrophotometer. Ascorbic acid (50 μ g/mL) and PBS (or PBS/DMSO, 1:0.063, for G-2 and G-3) were used as positive and negative controls, respectively. The blank of each sample/control was read at 517 nm before the addition of DPPH solution. A standard curve of DPPH (ranging from 0 to 60 μ M of DPPH) was read at the same wavelength to calculate its concentration relative to absorbance.

2.11. Macrophage cultures and immunomodulatory testing

The human monocyte THP-1 cell line was obtained from ATCC and cultured with supplemented RPMI 1640 medium (10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin, 2 mM L-glutamine and 0.05 mM β -mercaptoethanol). For differentiation into macrophages, THP-1 cells were seeded (5×10^5 cells/mL) in 24 well-plate with 100 ng/mL phorbol 12-myristate 13-acetate (PMA) and maintained for 48 h at 37 °C under 5% CO₂ in a humidified incubator.

Firstly, the glucans cytotoxicity (G-1, G-2, G-3) was evaluated in differentiated macrophages using 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide (MTT) protocol (Mosmann, 1983). Afterwards, the macrophages were washed with PBS and then replaced with serum-free medium containing LPS (0.05 μ g/mL) and subtoxic concentrations of the glucans. After 10 h of incubation, cells supernatants were collected and store at -20 °C until use.

Pro-inflammatory cytokines TNF- α (Tumour necrosis factor alpha), IL-1 β (Interleukin 1 beta) and IL-6 (Interleukin 6) were measured in the supernatants by BD Biosciences Human ELISA set (Aalst, Belgium) following the manufacturer's instructions. The quantification was calculated considering positive controls (cells stimulated with LPS) as a 100% cytokine secretion. The colour generated was determined by measuring the OD at 450 nm using a multiscanner autoreader (Sunrise,

Tecan). The assays were conducted in three independent experiments, in triplicated wells.

2.12. Inhibitory activity of tumoral cells growth

MDA-MB-231 breast cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (1%). The mammary non-tumorigenic epithelial cells MCF-10A was cultured in DMEM medium supplemented with 5% horse serum, 0.5 mg/mL hydrocortisone, 20 ng/mL recombinant EGF and 10 µg/mL insulin. Both cell lines were obtained from ATCC and they were maintained in a humidified atmosphere containing 5% CO₂ at 37 °C.

The normal (MCF-10A) and tumoral (MDA-MB-231) cells were seeded into 96-well plates (4×10^4 cells/mL) for 24 h to adhere. Later on, the cells were exposed to treatment with G-1, G-2, or G-3 (at 10, 50 or 250 µg/mL), for 24 and 48 h. The samples were solubilized in sterile PBS (G-1) or in a mixture of sterilized PBS:dimethyl sulfoxide (3:1) (G-2 and G-3) until complete solubilization. The presence of dimethyl sulfoxide at this concentration (1.25%) was not toxic for the cells (data not shown). Afterwards, the cell viability was determined by two different assays in separated plates: MTT test (according to Mosmann, 1983) and Live/Dead® Viability/Cytotoxicity kit (according to the manufacturer). PBS alone and the mixture of PBS:dimethyl sulfoxide (3:1) were used as control and the cell viability was expressed as a percentage of control cells. The assays were conducted in three independent experiments, in quadruplicated wells for MTT and sextuplicated wells for Live/Dead® Viability/Cytotoxicity kit. After the treatment MTT plates were read at 595 nm and Live/Dead plates were read in the InCell Analyzer 2000 Imaging System (GE, Healthcare, UK). Green and Red fluorescence intensity are recorded by the equipment from 4 fields in each well and the values of live and dead cells are calculated by the mean of each well.

2.13. Statistical analysis

Differences were evaluated at 95% confidence level ($P \leq 0.05$) using a one-way analysis of variance (ANOVA) followed by Tukey's or Bonferroni's Multiple Comparison test. Statistical analysis was performed using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA).

3. Results and discussion

3.1. Isolation and chemical characterization of the purified fractions

Several fractions were isolated from shiitake mushrooms (Fig. 1). After hot water extraction, the insoluble material yielded a crude polysaccharide extract (58.2% dw) containing 91.4% glucose and smaller amounts of mannose and galactose. HSQC-DEPT spectrum (Fig. 2a) of this fraction showed the presence of α - and β -D-glucans evidenced by signals relative to C-1/H-1 of α -D-Glcp at δ 99.2/4.98 and at δ 102.1/4.41 and 102.7/4.17 of β -D-Glcp. Signals at δ 82.6/3.56 and δ 85.7/3.38 indicated C-3 O-substitution, probably related to respectively α -D-Glcp and β -D-Glcp units. The (1 \rightarrow 3)-linkages are commonly found in β -glucans isolated from mushrooms (Synystya & Novak, 2013) and α -(1 \rightarrow 3)-linkages for glucans, although less observed, were previously detected in *Lentinula edodes* extracts (Morales, Smiderle, Villalva et al., 2019) and other mushrooms such as *Fomitopsis betulina* (de Jesus et al., 2018).

Based on literature and considering that the crude polysaccharide fraction might be a mixture of two or more glucans, the crude extract was further treated with mild alkaline conditions (de Jesus et al., 2018) to remove possible inter-molecular interactions between glucans. Then, two fractions were obtained: S-1 (soluble) and I-1 (insoluble) showing different signals in their NMR spectra. The spectrum of the soluble fraction S-1 (Fig. 2b) showed intense signals at δ 102.4/4.43; 102.6/

4.27 and 102.8/4.17, confirming β -configuration of D-Glcp and at 85.9/3.39 ppm relative to O-3 substitution of β -D-Glcp units; while I-1 spectrum (Fig. 2c) showed more intense signal at 99.2/5.00 ppm and 82.9/3.55 ppm indicating the presence of α -D-Glcp (1 \rightarrow 3)-linked. Small contaminations of other glucans were also noticed in both spectra, although the intensity of the main anomeric signals and (1 \rightarrow 3)-linked signals of each glucan (α - and β -) was an indicative of successful purification method.

To refine the samples purification, fraction S-1 was submitted to freeze-thawing process and divided into two fractions according to their solubility in cold water: soluble (G-1) and non-soluble (G-2). The monosaccharide composition of G-1 and G-2 were 87.6% and 81.4% glucose, respectively, and low contents of galactose and mannose (Supplementary Figs. 1 and 2). FT-IR spectra of both fractions showed characteristic bands of carbohydrates (Supplementary Fig. 4). Strong broad band between 3000 cm⁻¹ and 3500 cm⁻¹, centered at \sim 3400 cm⁻¹ indicate the presence of OH stretching vibration, and were observed in both spectra. The absorption observed at 1089 cm⁻¹ (for G-1) and at 1093 cm⁻¹ (for G-2) are characteristic of β -glucans (Kozarski et al., 2011; Synystya & Novak, 2014). G-1 presented also an evident absorption at 1436 cm⁻¹, which is representative of CH₂ (Synystya & Novak, 2014), and this suggest the presence of a linear glucan in G-1. A small peak was also observed in G-2 spectrum relative to CH₂ at 1456 cm⁻¹. Characteristic absorptions of protein was observed in both spectra at 1666 cm⁻¹ (G-1) and 1670 cm⁻¹ (G-2) (Kozarski et al., 2011).

FT-IR data corroborate the NMR results, the HSQC-DEPT of G-1 (Fig. 3a) suggested the major presence of a linear (1 \rightarrow 6)- β -D-glucan that was not previously reported in shiitake, but was detected in other species, such as *Agaricus* spp. (Smiderle et al., 2013). The signals corresponding to C-1/H-1 were observed at δ 102.7/4.26 and the inverted signals at δ 69.0/3.95 and 69.0/3.59, indicated the O-6 substitution, confirming the presence of a (1 \rightarrow 6)- β -D-glucan. Other four signals were evidenced corresponding to C-2/H-2 (δ 73.0/3.09), C-3/H-3 (δ 75.7/3.26), C-4/H-4 (δ 69.6/3.20) and C-5/H-5 (δ 74.7/3.37) of the main chain. However, the signals at 60.8/3.65 and 60.8/3.50 ppm indicated the presence of another polysaccharide with non-substituted CH₂ that could be traces of the (1 \rightarrow 3)- β -D-glucan observed in the other fractions. On the other hand, HSQC-DEPT of G-2 fraction (Fig. 3b) showed typical signals of a (1 \rightarrow 3)- β -D-glucan, branched at O-6 by β -D-Glcp units, commonly found in shiitake and other mushrooms (Ruthes et al., 2015). The intense signals at δ 102.6/4.46 and 102.8/4.17 were relative to C1/H1, at δ 85.9/3.39 indicated C3/H3 O-substituted, and at δ 68.1/3.93 and 68.1/3.50, confirmed CH₂-O-substituted of β -D-Glcp units. All the assignments were confirmed with literature data (Liu et al., 2014; Ruthes et al., 2013). G-1 and G-2 presented a mass-average molar mass (M_w) of 6,536 g/mol and 14,272 g/mol, respectively. M_w was calculated using $\partial n/\partial c$ value of 0.133 mL/g (Carbonero et al., 2006) and the recovery from SEC column was 100% for (1 \rightarrow 6)- β -D-glucan; while for (1 \rightarrow 3)-(1 \rightarrow 6)- β -D-glucan, $\partial n/\partial c$ value was 0.157 mL/g (Ruthes et al., 2013) and the recovery from SEC column was 70%.

Finally, when the insoluble fraction (I-1) was submitted to a second and stronger alkaline treatment, a residual fraction (not studied) and a highly insoluble fraction G-3 were obtained. The latter fraction included 100% glucose in its composition according to the GC-MS analysis (Supplementary Fig. 3). FT-IR spectrum of G-3 (Supplementary Fig. 4) presented similar absorption bands of the other two glucans, such as OH stretching vibration characteristic peaks at 3471 cm⁻¹, CH₂ absorption at 1463 cm⁻¹, however this sample did not show the typical band at \sim 1080 cm⁻¹ (relative to β -glucan). Instead, it was observed vibration ranging from 597 - 729 cm⁻¹, which indicates α -linkages (Kozarski et al., 2011; Synystya & Novak, 2014). Characteristic absorption of proteins was also observed for this sample at 1668 cm⁻¹.

More information about the chemical structure of G-3 glucan was obtained on its NMR spectrum (Fig. 3c), that showed main signals at 99.3/4.98 (C-1/H-1), 72.2/3.23 (C-2/H-2), 82.8/3.55 (C-3/H-3), 70.0/3.33 (C-4/H-4), 71.7/3.76 (C-5/H-5), inverted 60.5/3.58 and 60.3/4.42

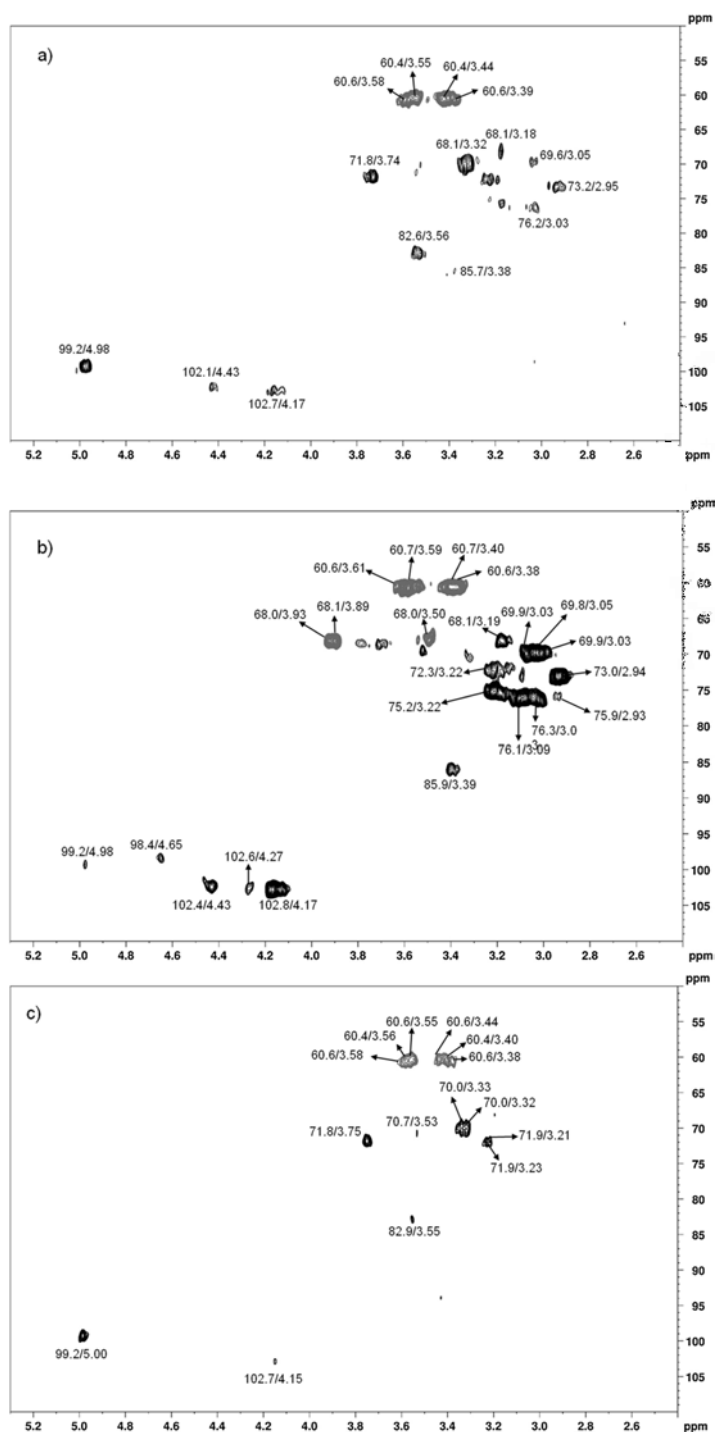


Fig. 2. HSQC-DEPT NMR spectra of crude polysaccharides fraction (a), S-1 (b) and I-1 (c). Experiment was performed in Me₂SO at 70 °C (chemical shifts are expressed in δ ppm).

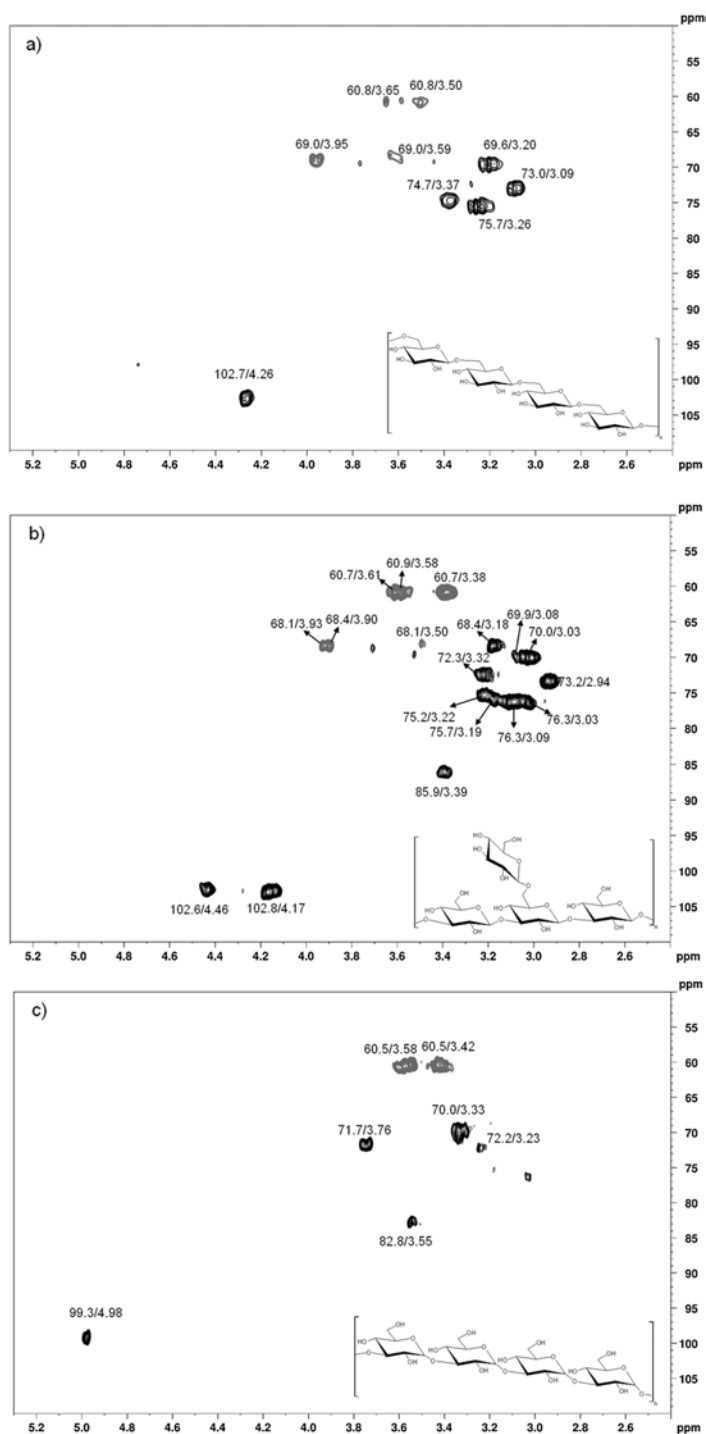


Fig. 3. HSQC-DEPT NMR spectra of G-1 (a); G-2 (b) and G-3 (c) fractions, and their respective chemical structures proposed. Experiments were performed in Me₂SO at 70 °C (chemical shifts are expressed in δ ppm).

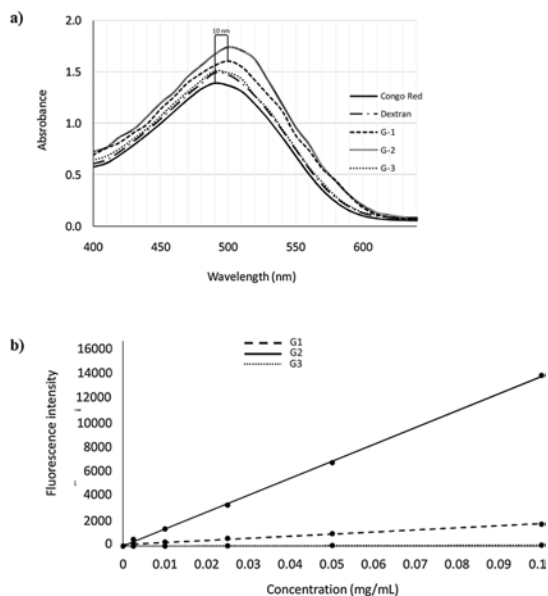


Fig. 4. a) Absorption spectra of Congo red (control) and Congo red with dextran, G-1, G-2 and G-3 and b) fluorescence intensity of G-1, G-2 and G-3 at different concentrations.

(C-6/H-6; CH₂) ppm, confirming that the purification process was efficient to isolate the (1→3)- α -D-glucan as performed previously by de Jesus et al. (2018). This fraction was also injected in SEC column, although, due to its high insolubility, the recovery from the column was 18%, and therefore the M_w value was not possible to be estimated.

Colorimetric determination with Congo red was used to determine the presence of triple helix conformation since Ogawa, Tsurugi, and Watanabe (1972) stated that polysaccharides with this tridimensional structure could form complex with Congo red, leading to a bathochromic shift of the maximum visible absorption (490 nm) of the Congo red spectrum. Dextran was used as random coil control and showed similar behaviour than Congo red solution, with no bathochromic shift. Fractions that contained the (1→6)- β -D-glucan (G-1) and (1→3)-(1→6)- β -D-glucan (G-2) displayed a bathochromic shift of 10 nm (Fig. 4a), suggesting triple helix conformations for such polysaccharides. On the other hand, fraction G-3 (Fig. 4a), which contained the (1→3)- α -D-glucan, showed no bathochromic shift, indicating random coil conformation such as the control of dextran. This bathochromic shift was also observed for a linear (1→3)- β -D-glucan isolated from *Cordyceps militaris* (Smiderle et al., 2014) and a branched (1→3)-(1→6)- β -D-glucan isolated from *Pleurotus ostreatus* (Palacios, García-Lafuente, Guillaumon, & Villares, 2012). An (1→4)- α -D-glucan obtained from *P. ostreatus* by the latter authors also presented no bathochromic shift as the (1→3)- α -D-glucan isolated in this study. These results confirm that different linkage types and anomeric configurations are strictly related to the tridimensional structure and, consequently to the therapeutic application of the glucans (Zhang et al., 2007).

Aniline blue/sirofluor is a fluorophore described and widely utilized for its specificity or preference to bind to (1→3)- β -D-glucans (Evans, Hoyne, & Stone, 1984; Gil-Ramirez et al., 2019). The branched β -D-glucan (G-2) exhibited intense fluorescence when compared to the linear β -D-glucan (G-1) and the linear α -D-glucan (G-3). The G-1 glucan showed slight fluorescence, while G-3 showed no fluorescence (Fig. 4b). Therefore, these results were in concordance with the NMR indications. It was possible to observe that the branched β -D-glucan (G-2) showed the highest fluorescence as also observed by Gil-Ramirez et al. (2019).

However, the fraction G-1, including mainly a (1→6)- β -D-glucan, showed a slight fluorescence, differing from results observed by other authors who detected no fluorescence for linear (1→6)- β -D-glucans (Gil-Ramirez et al., 2019). This might indicate that the fraction G-1 still contained a small amount of the branched (1→3)-(1→6)- β -D-glucan. Furthermore, the fluorescence absence of G-3 fraction confirmed that the (1→3)- α -D-glucan fraction excluded the presence of β -D-glucans.

3.2. HMGCR inhibitory activity

β -D-Glucans were pointed as hypocholesterolemic polysaccharides since they reduced cholesterol and bile acids concentrations in the intestinal lumen impairing their absorption by enterocytes. The precise mechanism is not completely elucidated but they might increase intestinal viscosity or/and scavenge small compounds within their complex structures leading to lower plasma cholesterol levels (Sima, Vannucci, & Vetrica, 2018). Moreover, Gil-Ramirez et al. (2017) found that certain mushroom β -D-glucans such as curdlan or schizophyllan were able to inhibit (*in vitro*) the activity of the 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR), the key enzyme in the biosynthesis of endogenous cholesterol and target of drugs such as statins. Tong et al. (2015) also observed reduction of HMGCR activity in hamsters liver when administrated barley β -D-glucans. Thus, the HMGCR inhibitory activity of the isolated glucans was evaluated and results indicated that they showed remarkable inhibitory activities (Table 1). The G-2 fraction containing the (1→3),(1→6)- β -D-glucan reduced the enzyme activity up to similar levels than reported for schizophyllan, a polysaccharide with similar structure (Gil-Ramirez et al., 2017). The linear β -D-glucan (G-1) and particularly the (1→3)- α -D-glucan (G-3) showed even higher inhibition capacities, higher than other α -D-glucans such as dextran (Gil-Ramirez et al., 2017).

3.3. DPPH scavenging capacity

The antioxidant activities of glucans and other polysaccharides are frequently related to some of their therapeutic benefits (Hong et al., 2013; Maity et al., 2017). Therefore, the free radical scavenging activities of the fractions G-1, G-2, and G-3 were also investigated using DPPH as radical. Only glucan fractions G-1 and G-2 showed scavenging effect on the DPPH radical, being G-1 the fraction with higher antioxidant activity (Fig. 5) with an IC₅₀ of 183.8 μ g/mL. This linear (1→6)- β -D-glucan showed higher chelating index than other fungal polysaccharides such as an heteropolysaccharide from *Pleurotus ostreatus*, which exhibited an IC₅₀ of 1.43 mg/mL (Zhang, Dai, Kong, & Chen, 2012); and a glucan-rich heteropolysaccharide from *Inonotus obliquus*, with an IC₅₀ of 1.3 mg/mL (Hu et al., 2016). Other authors evaluated the scavenging ability of glucan-rich extracts from *Agaricus bisporus*, *Pleurotus ostreatus*, and *Coprinus atramentarius* and observed lower scavenging activity (IC₅₀: ~5 mg/mL) for the three extracts in comparison to the linear (1→6)- β -D-glucan (Khan, Gani, Masoodi, Mushtaq, & Naik, 2017). No other isolated glucan was evaluated on DPPH assay.

3.4. Anti-inflammatory activity on immune cells

The immunomodulatory activity of the purified glucans was also

Table 1
HMGCR inhibitory activity (%) of G1, G2 and G3. (a-c) Different letters denote significant differences ($P < 0.05$) between samples.

Sample	HMGCR inhibition (%)
G-1	82.63 \pm 0.76 ^b
G-2	74.57 \pm 0.29 ^c
G-3	89.26 \pm 0.83 ^a

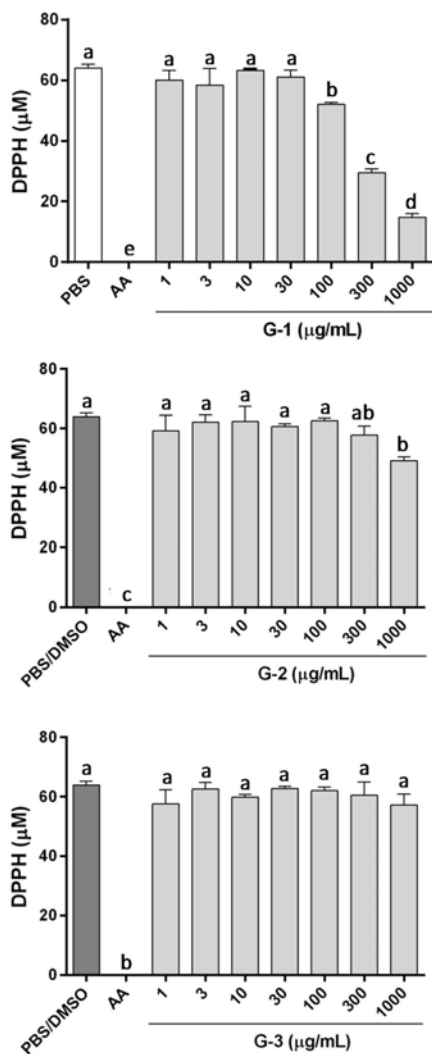


Fig. 5. Effects of glucans as DPPH radical scavengers. PBS or PBS/DMSO: negative control (vehicle). AA: positive control (ascorbic acid). Different letters (a–e) denote significant differences ($P < 0.05$) between samples.

tested as their capacity to reduce the secretion of pro-inflammatory cytokines in macrophages differentiated from THP-1 human monocytes cell line. The preliminary experiments to assess the glucans cytotoxicity indicated that when applied up to 10 μg/mL, the viability of THP-1 macrophages was not affected (data not shown). Thus, the immunomodulatory activity was tested in this subtoxic concentration. The THP-1 macrophages stimulated with LPS (positive control) exhibited a significant release of the three pro-inflammatory cytokines studied (TNF-α, IL-1β and IL-6) compared to non-stimulated cells (negative control) (Fig. 6). Addition of the glucans plus LPS did not reduce the amount of TNF-α liberated in the media, but significantly decreased IL-1β and IL-6 levels. Moreover, G-3 modulated IL-1β secretion reaching significantly lower values when compared to G-2 (43 and 26%, respectively), but all three glucans inhibited more than 42% the secretion of IL-6.

Previous reports testing mushroom glucans also showed anti-

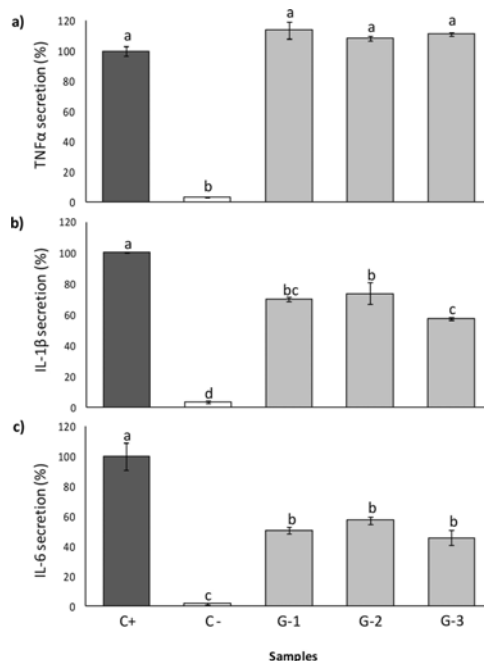


Fig. 6. Levels of a) TNF-α, b) IL-1β and c) IL-6 secreted by THP-1/M activated with LPS in presence of G-1, G-2 and G-3 (10 μg/mL). Positive control (C+): cells stimulated with LPS but in absence of extract. Negative control (C-): non LPS-activated cells. Different letters (a–d) denote significant differences ($P < 0.05$) between samples.

inflammatory effects. For instance, (1→6)-β-D-glucans from *Agaricus bisporus* and *Agaricus brasiliensis* were able to inhibit IL-1β and COX-2 expression when administered to LPS-activated THP-1 macrophages (Smiderle et al., 2013). Furthermore, a linear (1→3)-β-D-glucan isolated from *Cordyceps militaris* also inhibited the expression of IL-1β, TNF-α and COX-2 of THP-1 cells stimulated with LPS (Smiderle et al., 2014). Another linear (1→6)-β-D-glucan from *Pleurotus citrinopileatus* lowered the secreted levels of IL-6 and TNF-α differentiating macrophages stimulated with IFN-γ/LPS (Minato, Laan, van Die, & Mizuno, 2019). Comparing literature data with observed results, it seems that linear glucans, such as (1→6)-β-D-glucan (G-1) and (1→3)-α-D-glucan (G-3) produce more marked anti-inflammatory effects than the branched (1→6),(1→3)-β-D-glucans (G-2).

3.5. Cytotoxic effect on tumor cells

The antitumor activities of mushroom glucans are usually indirectly due to the stimulation of immune system that diminishes tumor resistance (Masuda et al., 2017; Zhang et al., 2007). However, the three isolated polysaccharides showed a direct effect on the viability of MDA-MB-231 breast tumor cells, as seen by MTT results (Fig. 7a, c and e). When the tumoral cells were separately treated with all fractions (G-1, G-2, G-3) a cytotoxic activity was noticed that was significant when applied mainly at 50 and 250 μg/mL, for 24 h and 48 h. However, when G-1, G-2 and G-3 were incubated with normal tissue breast cells (MCF-10A) no cytotoxic effect was observed (Fig. 7b, d and f).

The fraction G-1 containing mainly the linear (1→6)-β-D-glucan decreased the viability of tumor cell concomitant with the increase of applied concentration up to approx. 50% after 48 h of incubation when applied at 250 μg/mL. However, the branched (1→6),(1→3)-β-D-glucan (G-2) also diminished the viability approx. 50% (after 48 h)

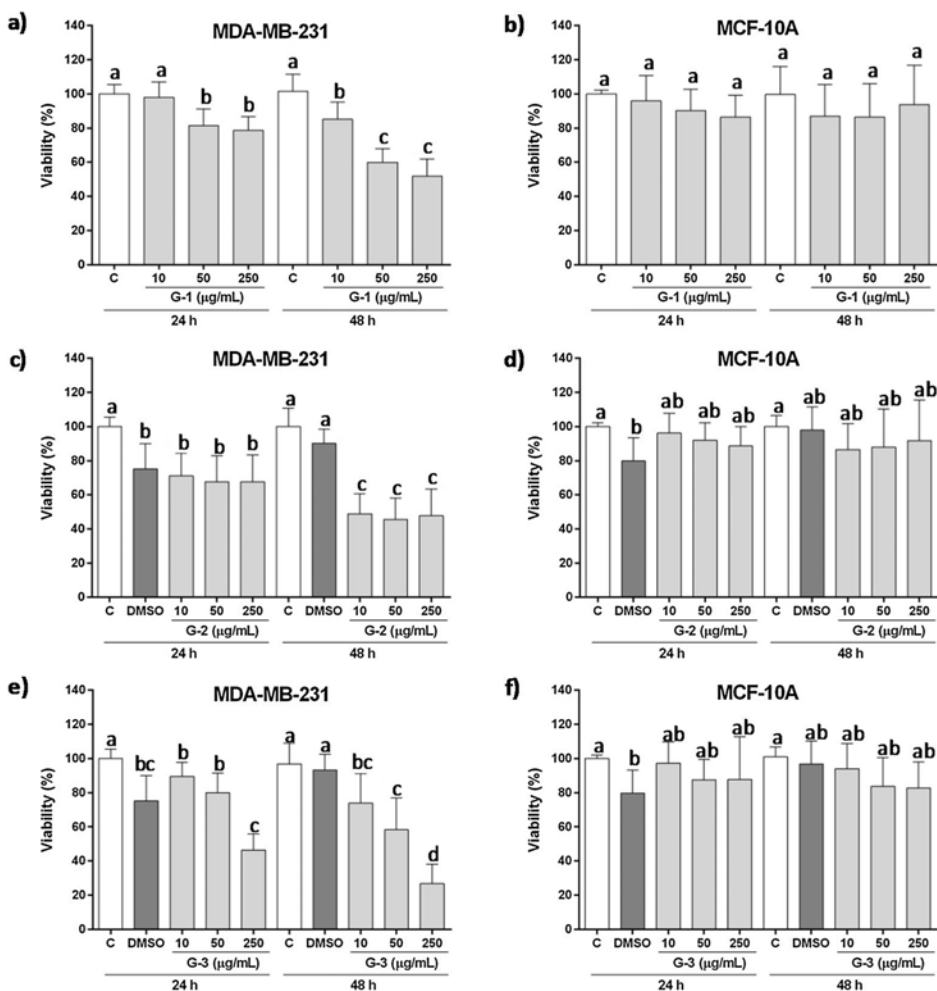


Fig. 7. Cell viability of MDA-MB-231 (tumor cell line, left) and MCF-10A (normal cell line, right) measured by MTT assay, after incubation with G-1 (a, b), G-2 (c, d) or G-3 (e, f) for 24 h and 48 h. C: medium plus PBS (vehicle); DMSO: medium plus dimethyl sulfoxide (1.25%). Different letters (a–c) denote significant differences ($P < 0.05$) between samples.

Table 2

Cell viability of MDA-MB-231 and MCF-10A measured by Live/Dead® Viability/Cytotoxicity kit, after incubation with G-1, G-2, or G-3 for 24 h and 48 h. Vehicle control: medium plus dimethyl sulfoxide (1.25%). Different letters (a–b) denote significant differences ($P < 0.05$) between samples for the same treatment time and cell line.

Cell Line: MDA-MB-231	24 h Treatment (μg/mL)				48 h Treatment (μg/mL)			
	10	50	250	Vehicle control	10	50	250	Vehicle control
G1	98,47 ± 1,32 a	99,27 ± 0,15 a	99,04 ± 0,22 a	99,30 ± 0,06 a	95,28 ± 3,33 b	98,62 ± 2,12 ab	99,36 ± 0,21 a	99,48 ± 0,11 a
G2	98,91 ± 0,22 a	95,78 ± 0,66 b	94,04 ± 1,40 b		98,83 ± 1,00 ab	98,04 ± 0,18 ab	98,53 ± 0,45 ab	
G3	99,19 ± 0,26 a	98,46 ± 0,24 a	93,17 ± 0,45 b		99,28 ± 0,06 ab	99,19 ± 0,07 ab	92,90 ± 1,71 b	

MCF-10A	24 h Treatment (μg/mL)				48 h Treatment (μg/mL)			
	10	50	250	Vehicle control	10	50	250	Vehicle control
G1	96,85 ± 0,15 b	97,15 ± 0,16 b	99,25 ± 0,10 a	97,73 ± 0,53 b	97,342 ± 0,3 b	97,78 ± 0,34 b	99,42 ± 0,12 a	97,43 ± 0,21 b
G2	97,40 ± 0,2 b	99,12 ± 0,34 a	99,93 ± 0,04 a		97,73 ± 0,31 b	98,94 ± 0,42 a	99,89 ± 0,02 a	
G3	96,85 ± 0,19 b	96,60 ± 0,28 b	97,89 ± 0,95 b		97,44 ± 0,57 b	97,17 ± 0,27 b	98,93 ± 0,58 a	

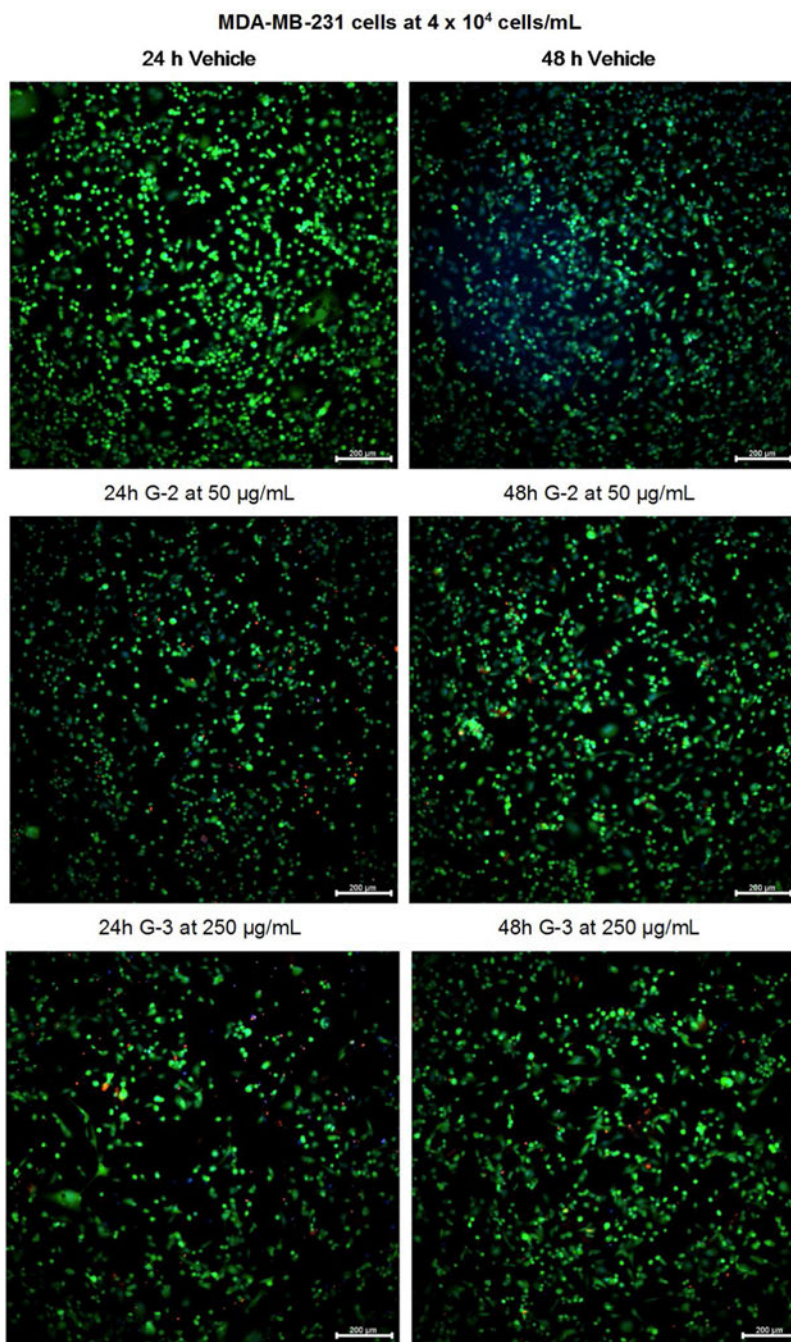


Fig. 8. MDA-MB-231 cells after 24 and 48 h of incubation (with the vehicle control, G-2 at 50 μ g/mL or G-3 at 250 μ g/mL) and addition of Live/Dead® Viability/Cytotoxicity kit. Blue fluorescence: cell nuclei; green fluorescence: live cells; and red fluorescence: dead cells. Pictures were taken by In Cell Analyzer.

independently of the tested concentration (10; 50; 250 μ g/mL). The highest cytotoxic activity was observed for the linear (1 \rightarrow 3)- α -D-glucan (G-3) where the noticed reduction was dependent of the concentration utilized. This glucan was able to reduce approx. 54% and 73% MDA-MB-231 cells viability after 24 h and 48 h of incubation, respectively,

when applied at 250 μ g/mL, being completely innocuous for MCF-10A cells.

When the cells treated, separately, with G-1, G-2 and G-3 were evaluated using Live/Dead Viability kit, which shows live cells with green fluorescence and dead cells with red fluorescence, the values of

cytotoxicity were less prominent than the results observed for MTT assay, however, the statistical analysis still showed significant toxicity to MDA-MB-231 cells treated with G-2 and G-3, in comparison with the cells treated only with vehicle (Table 2).

According to the results observed, the samples G-2 and G-3 were cytotoxic for the tumor cells (MDA-MB-231) after 24 h of treatment, at 250 µg/mL and G-3 exhibited the same effect after 48 h of treatment. By this technique, sample G-1 did not show any toxic effect to such cells. The non-tumorigenic cell line MCF-10A was not affected by the treatments and moreover it exhibited slight proliferation at the highest concentrations of the three glucans. Representative pictures can be observed on Fig. 8 and Supplementary Figs. 5–11.

4. Conclusions

The polysaccharide-enriched extract from *L. edodes* was a valuable source of bioactive glucans that were successfully isolated using easy and effective procedure with several steps such as alkaline extractions and freeze-thawing processes. Three glucan fractions that were separated contained mainly a linear (1→6)-β-D-glucan with low levels of (1→3)-β-D-glucan (G-1), a branched (1→3),(1→6)-β-D-glucan (G-2) and a linear (1→3)-α-D-glucan (G-3) according to GC-MS, FT-IR and NMR analyses. M_w of G-1 and G-2 was calculated as 6,536 g/mol ($\partial n/\partial c$: 0.133 mL/g) and 14,272 g/mol ($\partial n/\partial c$: 0.157 mL/g), respectively. Congo Red colorimetric assay indicated tridimensional conformation for G-1 and G-2 but not for G-3. The latter glucan emitted no fluorescence while G-2 showed high fluorescence intensity confirming its (1→3), (1→6)-β configuration. G-1 emitted significantly lower fluorescence indicating traces of (1→3)-β-D-glucans.

All the isolated glucans were able to inhibit HMGR *in vitro* and therefore, they might impair the cholesterol biosynthetic pathway. They also showed immunomodulatory activities although they did not induce significant variations on TNFα secretion by LPS-activated THP-1/M cells, they manage to lower IL-1β and IL-6 secretion. The glucans showed cytotoxic effects toward tumoral breast cells but they did not interfere with normal breast cells growth. The G-3 fraction showed the highest cytotoxic activity, reducing 73% viability after 48 h of incubation, when analyzed by MTT. The cytotoxic effect of G-3 was also observed when the cells treated with the glucan were analyzed by Live/Dead® Viability kit. However, it was observed that G-3 was unable to scavenge DPPH radical when applied at similar concentrations than G-1 and G-2 fractions. The results observed on this study suggest promising biological activities for the purified fractions and moreover, they demonstrated that different chemical structures observed for each glucan, such as α/β-configuration and branching degree may highly influence their solubility, tridimensional conformation and also their interaction with cells and consequently provide different biological outcomes. The most significant differences on the biological effects exerted by the three glucans were observed on the scavenging activity and the inhibition of tumor cell growth. Further investigations should be carried out to confirm if this difference may be related to chemical structure or physical conformation.

Acknowledgements

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Appendix A. Supplementary data


Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.carbpol.2019.115521>.

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Extraction of Bioactive Compounds Against Cardiovascular Diseases from *Lentinula edodes* Using a Sequential Extraction Method

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Three extraction methods were sequentially combined to obtain fractions from *Lentinula edodes* (shiitake mushrooms) containing bioactive compounds against cardiovascular diseases (CVDs). Fruiting bodies were first extracted with plain water, obtained residue was then submitted to supercritical fluid extraction (SFE) and remaining residue submitted to hot water extraction. Sequential design allowed reutilization of the nonextracted material as raw material for the successive extractions increasing extraction yields and separating interesting compounds. Obtained fractions contained different amounts of β -glucans, chitins, eritadenine, lenthionine, ergosterol, proteins/peptides and phenolic compounds conferring them different bioactivities. Water soluble fractions showed high antioxidant activities (ABTS⁺⁺ and DPPH[•] scavenging capacity and reducing power), they were also able to inhibit one of the main enzymes involved in hypertension (angiotensin-I converting enzyme) and the key enzyme of cholesterol metabolism (3-hydroxy-3-methylglutaryl coenzyme A reductase). The latter inhibitory activity was also noticed in SFE extracts although ergosterol and other lipid-like molecules were isolated. Dietary fibers were separated in the third extraction. Therefore, with this sequential extraction procedure bioactive compounds against CVDs can be selectively separated from a single batch of shiitake powder. © 2018 American Institute of Chemical Engineers *Biotechnol. Prog.*, 34:746–755, 2018

Keywords: supercritical CO₂ extraction, shiitake mushrooms, cholesterol, antioxidant activity, HMGCR inhibition

Introduction

Cardiovascular diseases (CVDs) are still one of the leading causes of mortality in developed countries. CVDs are associated with multiple factors such as high triglycerides and low density lipoprotein cholesterol (LDLc) levels, LDL oxidation, increased platelet aggregation, hypertension, and smoking.^{1,2}

Nowadays, functional foods containing phytosterols or β -glucans are being commercialized to reduce low to moderate hypercholesterolemia because these compounds showed the ability of impairing exogenous cholesterol absorption. Hypotensive foods are also marketed containing specific peptides able to inhibit the angiotensin I converting enzyme (ACE)³ and many juices and functional drinks indicate that they contain many compounds with high antioxidant properties. These compounds might inhibit LDL oxidation, prevent atheroma plaque formation, and so forth, contributing to reduce the risk of CVDs. However, a more effective novel food

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may be designed if all compounds are combined and, besides those inhibiting cholesterol absorption, other compounds are incorporated such as those reducing the risk of CVDs by different mechanisms of action.⁴

Edible mushrooms are natural sources of hypocholesterolemic compounds, such as fungal sterols (ergosterol and derivatives) and polysaccharides (β -glucans, chitins, etc.) that according to in vitro experiments might inhibit cholesterol absorption and biosynthesis.^{4–7} Eritadenine (2(*R*),3(*R*)-dihydroxy-4-(9-adenyl) butanoic acid) from *Lentinula edodes* (shiitake mushrooms) was also able to lower cholesterol levels by acting as an inhibitor of the S-adenosyl-L-homocysteine hydrolase involved in the hepatic phospholipid metabolism.^{8,9} When lard was supplemented with extracts containing these compounds and fed to mice, they succeeded to avoid the increasing of cholesterol levels noticed in control mice fed only with lard.¹⁰

Moreover, water extracts obtained from edible mushrooms such as *Hypsizygus marmoreus* and *Lactarius camphorates* were also able to inhibit ACE because of their hypotensive peptides^{11,12} and lenthionine (1,2,3,5,6-pentahiepane), an organosulfur compound responsible for the characteristic flavor of shiitake mushrooms, inhibited platelet aggregation.¹³ Water and hot water extracts from other mushroom species also showed interesting antioxidant activities that correlated to their levels of ergothioneine and phenolic compounds.^{14–16}

Several methods to isolate or extract each type of those previously mentioned compounds have been already reported. Different β -glucans types are usually isolated by alkali/acid treatments, hot water (120°C, 20 min), microwave, pressurized solvent, or ultrasound assisted extractions,^{17–19} chitins needed more drastic treatments,²⁰ and sterols were isolated using mixtures of organic solvents or supercritical CO₂ extractions.²¹ However, all these methods were optimized to use mushrooms as raw material and for each extraction, a new batch of powdered mushrooms should be used. In this work, a sequential extraction method was designed to extract from the same batch of mushroom powder a few fractions enriched in different bioactive compounds against CVDs. The residue remaining after one extraction was used to extract other compounds at the following extraction step instead of using different batches of mushroom powder for each type of compound.

Materials and Methods

Biological material

Dry *Lentinula edodes* S. (Berkeley) mushrooms were obtained in a local market (Madrid, Spain). Fruiting bodies were ground as a whole or divided into caps and stipes until a fine powder was obtained and stored at –20°C as indicated by Ramirez-Anguiano et al., 2007¹⁶ (they were used to analyze separated tissues). Larger amounts of powdered shiitake mushrooms were also purchased from Glucanfeed S.L. (La Rioja, Spain). Obtained powder showed a particle size lower than 0.5 mm, and moisture content lower than 5% and it was stored in darkness at –20°C until further use.

Reagents

Solvents as hexane (95%), chloroform (HPLC grade), methanol (HPLC grade), acetonitrile (HPLC grade) were obtained from LAB-SCAN (Gliwice, Poland) and absolute

ethanol, sodium carbonate (Na₂CO₃) and sulfuric acid (H₂SO₄) from Panreac (Barcelona, Spain). Potassium hydroxide (KOH), ascorbic acid, 2,6-Di-tert-butyl-*p*-cresol (BHT), bovine serum albumin (BSA), acetylacetone, *p*-dimethylaminebenzaldehyde, Trizma base, HCl (37%), trifluoroacetic acid (99%), phenol, Folin Ciocalteu's phenol reagent, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), potassium persulfate, Angiotensin-I Converting Enzyme (ACE) (5 UN/mL), zinc chloride (ZnCl₂) solution (0.1 M) as well as hexadecane, ergosterol (95%), D-glucose, D-glucosamine hydrochloride, gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium phosphate monobasic dihydrate (NaH₂PO₄ · 2H₂O), sodium phosphate dibasic anhydride (Na₂HPO₄), potassium ferricyanide (K₃[Fe(CN)₆]), ferric chloride (FeCl₃) and Trolox were purchased from Sigma-Aldrich Quimica (Madrid, Spain). CO₂ was supplied by Air-Liquid, S.A. (Madrid, Spain). D-Eritadenine (90%) was acquired from Sy Synchem UG & Co. KG (Felsberg, Germany), lenthionine (80%) from Cymit (Barcelona, Spain) and N-(2-aminobenzoyl)glycyl-4-nitro-L-phenylalanil-L-proline (Abz-Gly-Phe(NO₂)-Pro) from Bachem Feinchemikalien (Bubendorf, Switzerland). All other reagents and solvents were used of analytical grade.

Sequential extractions

A method to obtain different bioactive fractions from powdered shiitake mushrooms was optimized based on three successive extractions (Figure 1). First (Step A), mushroom powder was mixed with water (50 g/L) at room temperature (RT) and vigorously stirred during 1 minute. Afterwards, the mixture was centrifuged (7 min, 7,000 rpm, 10°C) in a Heraeus Multifuge 3SR+ centrifuge (Thermo Fisher Scientific, Madrid, Spain). Obtained supernatant (considered extract ExA) was separated from the residue (ReA) and both fractions were freeze dried in a LyoBeta 15 lyophilizer (Telstar, Madrid, Spain).

Second (Step B), freeze-dried ReA (253 g) was ground, sieved until particles size <0.5 mm and submitted to supercritical fluid extraction (SFE). ReA was mixed with 1.9 kg of 5 mm diameter stainless steel spheres (a ratio 1:1 (v/v) extract:spheres) in a 2 L extraction cell connected to a SFE pilot-scale plant (model SF2000, TharTechnology, Pittsburgh, PA). Pressurized CO₂ was forced to reach supercritical state and injected in the loaded extraction cell. Extracted material was collected in two different separators (S1 and S2) each of 0.5 L capacity with independent control of temperature and pressure. The extraction vessel had a ratio of 5.5 height/diameter (a detailed explanation of the experimental device can be found at Garcia-Risco et al., 2011²²). Extraction was carried out at 35 MPa and 40°C. Pressure of S1 and S2 was maintained at 10 and 6 MPa, respectively, and temperature in both of them was 40°C. The CO₂ flow was set at 3.6 kg/h and during the total extraction time (3 h) and it was recirculated. Extracted compounds were precipitated in both separators and at the end of the extraction process, the fractions were dragged with ethanol and immediately submitted to concentration until dryness on a rotary vacuum evaporator. Dried extract (ExB) was stored at –20°C until further analysis and nonextracted residual material (ReB) was separated from steel spheres by sieving.

Finally (Step C), ReB (100 g/L) was submitted to hot water extraction (98°C) in a 0.5 L flask during 1 h under vigorous stirring and reflux using a glass steam condenser.

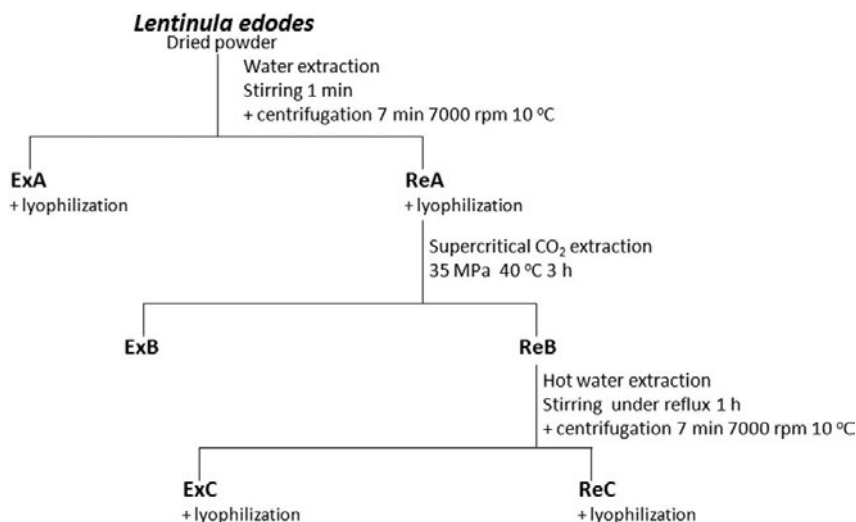


Figure 1. Outline of the sequential extraction method utilized to obtain different extracts from shiitake mushrooms.

The soluble (ExC) and nonsoluble (ReC) fractions were separated by centrifugation (7 min, 7,000 rpm, 10°C) and freeze dried.

Carbohydrates determinations

The total carbohydrate content of shiitake mushroom samples or obtained extracts was determined by the phenol-sulphuric acid method adapted from Dubois et al. (1956).²³ Briefly, samples (1 mg) were mixed with 1 mL of MilliQ water and stirred during 2 min. The mixtures (25 µL) were added to a 96-well plate, plus 25 µL of 5% phenol solution (w/v) and 125 µL of concentrated H₂SO₄. Afterwards, the plate was sealed and incubated in a water bath at 80°C for 30 min. Samples absorbance was read using a M200 Plate Reader (Tecan, Männedorf, Switzerland) at 595 nm. A standard curve of D-glucose (0.032–0.8 mg/mL) was used for quantification.

Chitin content was determined according to Smiderle et al. (2017).¹⁸ First, samples were hydrolyzed with 6 M HCl at 100°C for 2 h and adjusted to pH 10.0 after cooling down. Then, hydrolyzed samples (250 µL) were treated as described by Rementeria et al. (1991).²⁴ Samples absorbance was measured at 530 nm using an Evolution 600 UV-vis (Thermo Fisher Scientific, Spain) spectrophotometer. A glucosamine hydrochloride standard curve was used for quantification.

The β-glucan content of the obtained mushroom samples and extracts (50 mg) was evaluated by a β-glucan determination kit specific for mushrooms and yeasts (Megazyme®, Biocom, Barcelona, Spain) following the instructions of the user's manual.

Total phenol content and proteins or peptides determination

The total phenol concentration of samples (10 mg) was determined by the Folin-Ciocalteu method according to the

procedure of Ramirez-Anguiano et al. (2007).¹⁶ Gallic acid was used as standard for quantification.

The total protein concentration of the samples (10 mg/mL) was determined using the Bradford method reagents (Sigma-Aldrich, Madrid, Spain) according to the Instruction Manual. To determine the amount of peptides, extracts were solubilized in water (100 mg/mL) and submitted to centrifugation (14,000 rpm, 30 min) using Amicon Ultra filter devices with Ultracel 3K membrane (Millipore, Billerica) obtaining a filtrate (< 3 kDa) and a concentrate (> 3 kDa). The latter fraction was submitted to a second centrifugation (14,000 rpm, 20 min) using Nanosep centrifugal device with Omega 10 K membrane (Pall Life Sciences, New York), obtaining a filtrate (fraction with MW between 3 and 10 kDa) and a concentrate (> 10 kDa). The obtained fractions were freeze dried and mixed with the Bradford reagent as described above. BSA was used as standard (0.0125–0.5 mg/mL) for protein quantification.

Eritadenine and lenthionine determination by HPLC-DAD

Eritadenine was extracted from the samples and analyzed following the procedure of Afrin et al. (2016)²⁵ with modifications. Briefly, samples (1 g) were mixed with 10 mL of 60% ethanol (v/v) and stirred for 2 min. The mixture was centrifuged (15 min, 7,000 rpm, 10°C) and the supernatant was collected. Afterwards, 10 mL of 60% ethanol (v/v) was added for a second extraction and both supernatants were pooled together and submitted to vacuum filtration. The filtrate was concentrated on a rotary vacuum extractor at 60°C until dryness. Identification and quantification of eritadenine were carried out using a C18 Spherisorb ODS2 4 × 250 mm analytical column with a 5 µm particle size (Waters, Mississauga, Ontario, Canada) coupled to an HPLC system (Pro-Star 330, Varian, Madrid, Spain) with PDA detector (Pro-Star 363 module, Varian, Madrid, Spain). Samples were dissolved in mobile phase (5 mg/mL) and they were injected (10 µL) and developed under a constant flow (0.5 mL/min)

and an isocratic mobile phase of water:acetonitrile (98:2, v/v 1% TFA). Eritadenine was quantified at 260 nm using a commercial standard. The compound eluted at 11.6 min and showed the characteristic eritadenine UV-spectrum.

Lenthionine determination was carried out according to the procedure of Hiraide et al. (2010)²⁶ with slight modifications. Basically, samples (50 mg) were mixed with 1 mL of 0.2 M Tris-HCl buffer (pH 8.0) and stirred for 1 h. Afterwards, methanol (0.5 mL) was added, stirred for 2 min and centrifuged (14,000 rpm 5 min). The obtained supernatant was collected and the residue submitted to extraction twice. Supernatants were pooled together, diluted with 2.5 mL MilliQ water and filtered using a syringe through a 0.45 µm pore size filter. The filtrate was applied to an ODS cartridge (Waters, Missisagua, Ontario, Canada) preactivated with methanol following manufacturer's instructions. The cartridge was washed with 1 mL 30% methanol (v/v) and lenthionine was eluted with 1 mL 65% methanol (v/v). Obtained eluate (50 µL) was injected into an HPLC-DAD system (the same column and equipment than above described) and developed using an isocratic mobile phase (65% methanol v/v), a constant flow (0.7 mL/min) and temperature (45°C). Lenthionine (retention time 10.4 min) was quantified at 230 nm and identified using a lenthionine commercial standard.

Ergosterol determination by GC-MS-FID

Fungal sterols from samples were evaluated following the procedure described by Gil-Ramirez et al. (2013).²¹ The unsaponified fractions obtained (6 mg/mL) were injected into an Agilent HP-5ms capillary column (30 m × 0.25 mm i.d. and 0.25 µm phase thickness). The column was connected to a 7890A System gas chromatograph (Agilent Technologies, Santa Clara, CA), comprising a split/splitless injector, an electronic pressure control, a G4513A autoinjector, a 5975C triple-axis mass spectrometer detector and a GC-MS Solution software. The injector and detector conditions as well as the column temperature program were those described by Gil-Ramirez et al. (2013).²¹ Ergosterol was used as standard and hexadecane (10% v/v) as internal standard for quantification.

Antioxidant activities

Mushroom powder and obtained extracts were dissolved in water (0.02–0.5 mg/mL) and assayed for their ABTS⁺⁺ scavenging activity assay. ABTS radical was chemically generated using potassium persulfate and ABTS⁺⁺ scavenging activity was analyzed spectrophotometrically according to Re et al. (1999),²⁷ measuring changes in absorbance (734 nm) at several concentrations after 15 min of incubation at room temperature in darkness. Similarly, samples were also dissolved in methanol (0.02–0.5 mg/mL) and mixed with DPPH[•] (76 µM) to determine their scavenging capacity according to Mau et al. (2001).²⁸ Absorbance at 517 nm was recorded at several concentrations after 15 min incubation at room temperature in darkness. For both radicals the IC₅₀ was established using the linear correlation obtained with increasing sample concentrations and compared with Trolox to express the results as their TEAC values (trolox equivalent antioxidant capacity).

The ferric ion reducing power of the extracts was evaluated according to the method of Oyaizu (1986).²⁹ Samples were dissolved in 200 mM sodium phosphate buffer (0.1–10 mg/mL) and treated as described by Mau et al. (2005).³⁰

Absorbance increase was recorded at 700 nm using several concentrations to estimate their EC₅₀. Afterwards, it was expressed as TEAC values to be able to compare with the other antioxidant activities.

HMGCR inhibitory activity

The obtained extracts were solubilized in water, ethanol:water (1:4) or assay buffer (50 mg/mL) and applied (20 µL) into a 96-wells plate. Their inhibitory activity was measured using the commercial HMGCR (3-hydroxy-3-methylglutaryl coenzyme A reductase) activity assay (Sigma-Aldrich, Madrid, Spain) according to the manufacturer's instructions by monitoring their absorbance change (340 nm) at 37°C using a 96-wells microplate reader BioTek Sinergy HT (Bio-Tek, Winooski). Pravastatin was utilized as a control for positive inhibition.

ACE inhibitory activity

The Angiotensin-I Converting Enzyme (ACE) inhibitory activity of the obtained extracts was evaluated using the fluorimetric method described by Sentandreu and Toldra (2006)³¹ with slight modifications. Basically, 40 µL of different samples dilutions were added to a 96-wells plate, followed by addition of 160 µL of Abz-Gly-Phe(NO₂)-Pro (0.45 mM) and 40 µL of ACE working solution (0.04 U/mL). The plate was incubated during 1 h at 37°C measuring the generated fluorescence with excitation and emission wavelengths, respectively, 355 and 405 nm. Milli Q water was utilized as a control for negative inhibition.

Statistical analysis

Differences were evaluated at a 95% confidence level ($P \leq 0.05$) using a one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison test. Statistical analysis was performed using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA). Correlation between different variables was evaluated by computing Pearson correlation coefficient and determination coefficient ($P \leq 0.05$).

Results and Discussion

Selection of the starting material

The distribution of compounds with beneficial activities against CVDs within the *Lentinula edodes* fruiting body was investigated as a preliminary study to elucidate whether it was more convenient to use the complete mushroom or perhaps only caps or stipes as raw material for the extractions. However, when the amount of the main bioactive compounds was determined (Table 1), no significant differences were noticed between tissues. They contained approximately 40% (w/w) carbohydrates and most of them were β-glucans since chitins were found in lower concentrations. Ergosterol and the other bioactive compounds showed levels similar to previous studies^{16,25,32} being lenthionine the compound found in the lowest concentration (0.15 mg/g) but also similar to other publications.^{13,26}

The lower part of *L. edodes* stipes (the one is in contact with the cultivation substrate) is considered as a by-product and it is usually discarded during harvesting and not commercialized. However, the stipes showed similar concentrations than the complete fruiting body not only of the

Table 1. Total Carbohydrates (TC), β -glucans (β G), Chitins (CH), Total Proteins (PR), Eritadenine (EA), Lenthionine (LT), Ergosterol (ER), and Total Phenolic Compounds (PH) Levels of Shiitake Fruiting Bodies and Two Separated Tissues (Indicated Values are w/w)

	TC (g/100g)	β G (g/100g)	CH (g/100g)	PR (g/100g)	EA (mg/g)	LT (mg/g)	ER (mg/g)	PH (mg/g)
Whole	40.67 \pm 0.65	29.32 \pm 1.04	6.03 \pm 0.29	13.42 \pm 0.22	1.43 \pm 0.13	0.15 \pm 0.01	2.40 \pm 0.02	9.27 \pm 0.05
Cap	39.72 \pm 2.42	29.89 \pm 1.55	5.96 \pm 0.87	13.81 \pm 0.12	1.13 \pm 0.01	0.13 \pm 0.01	2.24 \pm 0.04	9.68 \pm 0.50
Stipe	42.37 \pm 1.42	32.01 \pm 1.72	6.28 \pm 1.09	12.90 \pm 0.08	1.12 \pm 0.11	0.11 \pm 0.01	2.32 \pm 0.05	8.95 \pm 0.43

No significant differences ($P \leq 0.05$) were found between different samples for the same component.

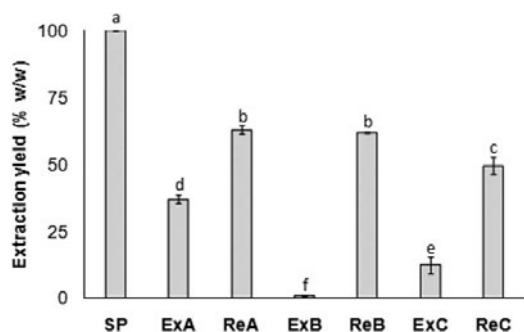


Figure 2. Extraction yields obtained after each step of the sequential extraction method expressed as percentage (w/w) referred to the initial amount of shiitake powder (SP).

Different letters (a–f) showed statistical significance ($P \leq 0.05$) between different extractions.

bioactive molecules but also of other nutrients such as proteins or carbohydrates. This observation indicated that the wasted stipes could be used as source of bioactive compounds as well as the complete fruiting bodies being an alternative for valorization of these wastes. Nevertheless, since the fruiting bodies were more easily available in larger concentrations the following experiments were carried out using the complete mushrooms.

Sequential extraction yields

The sequential extraction method was designed to optimize the use of shiitake mushrooms or their by-products (as they showed similar composition) as material to extract bioactive compounds. The idea was to consecutively use the residue of one extraction to carry out the next one to maximize the number of compounds that could be selectively isolated in each step avoiding the use of larger amounts of mushroom powder for independent extractions.

Water extraction (at room temperature) was selected as first extraction step because some of the bioactive compounds that could be solubilized in this medium were not thermostable (see later for details). Then, the lipid fraction could be extracted with SFE using moderate temperatures (as second step) and in the third step hot water could be used to extract heat resistant molecules such as dietary fibers improving the intestinal bioavailability (for specific β -glucans) and making them more accessible for colonic microbiota.^{33,34}

With the first extraction, water extracted 37 g from 100 g (w/w) of the mushroom powder (Figure 2). This method was previously used to obtain water-soluble polysaccharides³⁵ and other compounds of lower molecular weight.³⁶ The lowest yield (1.1%) was obtained with supercritical CO₂

extraction of the generated residue (1.7% of ReA) since it mostly extracted lipophilic compounds (ExB) and mushrooms have a low lipid content. Moreover, supercritical CO₂ exhibit a high selectivity enabling high recoveries of specific fatty acids, sterols and derivatives from fungal or other matrices.³⁷ Nevertheless, the obtained yield was similar to those described in previous publications where SFE extractions were carried out directly from fruiting bodies^{38,39} indicating that SFE extractions could be also carried out using the residues obtained after the first extraction step. The last step, a hot water extraction (98°C), generated a fraction (ExC) containing 20% (w/w) of ReB (12% of the initial mushroom powder) leaving still a large insoluble fraction (ReC) encompassing 49.5% of the starting material. This procedure is usually utilized to extract certain polysaccharides from the rest of nonsoluble dietary fibres that remain in the residual fraction.^{40,41}

Distribution of bioactive compounds within the obtained fractions

The levels of the different fungal polysaccharides were evaluated in all the obtained fractions. Results indicated that the water insoluble fractions contained higher levels of total carbohydrates, β -glucans and chitins than water-soluble or SFE fractions (Figure 3a). The mushroom powder contained 40.7% (w/w) total carbohydrates where 29.3% of them were β -glucans and 6.0% chitins suggesting that the remaining low percentage (~5%) should include oligosaccharides, sugars and perhaps small amounts of α -glucans or other heterosaccharides. Total carbohydrates values were in concordance with the literature.⁴² β -glucans and chitins were slightly higher than some reports^{43,44} but in the same range than others.^{45–47} Differences might be due to the different methodologies utilized for their determination.

The water extract (ExA) contained a lower content of total carbohydrates and more than half of them were probably sugars and oligomers since they are easily solubilized in water and their β -glucan content was also lower than in the mushroom powder (10.8%). Although in low amounts, chitins were also detected but they might be degradation products or low molecular weight derivatives from chitins since the latter compounds are completely insoluble in water. These derivatives were also noticed in other reports^{17,20} and they might be involved, together with the water soluble β -glucans, in the interesting biological activities noticed in mushroom water extracts. Water soluble β -glucans, α -glucans and fucmannogalactans were pointed as compounds potentially involved in the HMG-CoA reductase inhibitory activity noticed in vitro for water soluble extracts.³⁶ Moreover, chitin oligomers (water-soluble low molecular weight chitin (LMWC) and chito oligosaccharides (COs) derivatives) could be involved in the hypocholesterolemic properties noticed for these extracts.⁴⁸

ReA and ReB showed a very similar carbohydrates profile including approximately 39–40% β -glucans and 7–8% chitins

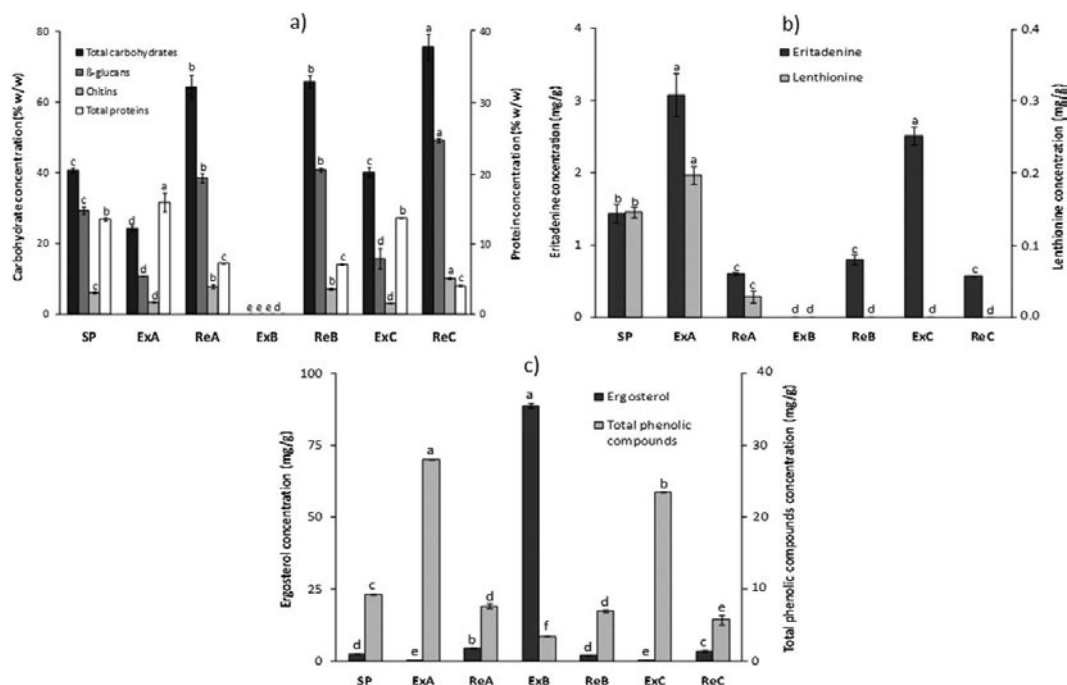


Figure 3. Distribution of bioactive compounds within the extracts obtained from shiitake powder expressed as concentrations within each fraction.

Levels of (a) total carbohydrates, β -glucans, chitins, and proteins, (b) eritadenine and lenthionine, and (c) ergosterol and total phenolic compounds. Different letters (a–f) showed statistical significance ($P \leq 0.05$) between different samples for the same compound.

indicating that the supercritical CO_2 extraction did not significantly influenced the polysaccharide composition of the residue obtained after water extraction and none of them was extracted.

Alkaline/acid or hot water extractions are usually required to achieve fractions with high polysaccharides yields.³⁵ The hot water extraction did not succeed to separate β -glucans from chitins. The β -glucans extracted with hot water accounted for approx. less than half of the total carbohydrates noticed in ExC. As probably most of the oligosaccharides and monosaccharides were previously extracted in the ExA, the rest of carbohydrate content could be due to α -glucans or other heteropolysaccharides characteristic from *L. edodes* that were also extracted. Shiitake β -glucans such as lentinan, are usually isolated using hot water extractions although their yields could be enhanced if alkaline media are used. However, alkalis were not recommended since they interfere with the lentinan 3D structure, essential for its biological activities.⁴⁹ Hot water soluble polysaccharides were suggested to decrease the oxidative damage related to hypercholesterolemia and modulating immune system.^{50–52}

Furthermore, approximately 2/3 of the carbohydrates present in the residue ReC were β -glucans, and chitins (10.2% w/w) that remained being part of the insoluble fractions. They were not extracted neither with cold/hot water or supercritical CO_2 since their concentrations in all the analyzed residues were almost the same. Their complex-forming capacity and polymeric structure are responsible for their extremely low solubility in many simple solvents.⁵³ However, this characteristic confers them the ability to act as

dietary fibres remaining undigested in the intestine and acting on the colonic flora as prebiotic or as hypocholesterolemic fibers.^{48,54}

The total protein content measured in shiitake mushroom was in the range of previously reported studies since they might change from approximately 13 to 23% depending on the cultivation conditions, developmental stage or commercial strain.^{55–57} The highest protein content (Figure 3a) was obtained in ExA, indicating that a considerable amount of Shiitake proteins might be easily extracted with cold water, probably because free proteins, peptides and amino acid derivatives are included in this fraction. Indeed, in this fraction higher amounts of small proteins and peptides (8.57 mg/g) (with molecular weight (MW) between 10 and 3 kDa) than larger proteins (5.88 mg/g) (MW > 10 kDa) were noticed. It also contained a low amount of peptides and N-containing compounds with MW < 3kDa (0.83 mg/g). But, proteins might be also bound to polysaccharides as glycoproteins or proteoglycans impairing their extraction, therefore, other proteins were found in the residues. The high pressure (and/or mild temperature) utilized during SFE extraction seemed to partially denature proteins since ReB protein levels were not the sum of those noticed in the fractions from the following extraction step (Table 2). Nevertheless, the ExC obtained still contained high protein levels and only low amounts remained in ReC (Figure 3a) suggesting that hot water enhanced extraction of many more proteins or different ones from those found in ExA. Nevertheless, the temperature selected might have also influenced proteins structure generating breaking down products from large

Table 2. Distribution of Bioactive Compounds Within the Obtained Fractions Expressed as Percentage of Initial Dry Mushroom Powder (Taking into Consideration the Obtained Yields)

	TC (g/100 g)	β G (g/100 g)	CH (g/100 g)	PR (g/100g SP)	EA (mg/g)	LT (mg/g)	ER (mg/g)	PH (mg/g)
SP	40.67 \pm 0.34 ^a	29.32 \pm 1.04 ^a	6.03 \pm 0.29 ^a	13.42 \pm 0.22 ^a	1.43 \pm 0.13 ^a	0.15 \pm 0.01 ^a	2.40 \pm 0.02 ^a	9.27 \pm 0.05 ^b
ExA	11.46 \pm 0.37 ^d	5.10 \pm 0.09 ^d	1.58 \pm 0.08 ^d	5.86 \pm 0.49 ^b	1.46 \pm 0.14 ^a	0.09 \pm 0.01 ^b	0.03 \pm 0.01 ^e	10.35 \pm 0.01 ^a
ReA	33.84 \pm 1.80 ^b	20.32 \pm 0.73 ^b	4.11 \pm 0.26 ^b	4.53 \pm 0.01 ^c	0.32 \pm 0.01 ^{bc}	0.01 \pm 0.01 ^c	2.12 \pm 0.11 ^b	4.84 \pm 0.20 ^c
ExB	n.d. ^c	n.d. ^f	n.d. ^f	n.d. ^c	n.d. ^c	n.d. ^c	0.79 \pm 0.01 ^d	0.04 \pm 0.00 ^c
ReB	34.07 \pm 0.94 ^b	21.17 \pm 0.24 ^b	3.66 \pm 0.10 ^b	4.36 \pm 0.04 ^c	0.41 \pm 0.03 ^{bc}	n.d. ^c	1.08 \pm 0.01 ^c	4.33 \pm 0.10 ^c
ExC	9.06 \pm 0.31 ^d	3.53 \pm 0.65 ^c	0.86 \pm 0.03 ^c	1.63 \pm 0.01 ^d	0.57 \pm 0.03 ^b	n.d. ^c	0.06 \pm 0.01 ^e	2.91 \pm 0.01 ^d
ReC	22.14 \pm 1.05 ^c	14.38 \pm 0.15 ^c	2.29 \pm 0.06 ^c	2.00 \pm 0.04 ^d	0.17 \pm 0.00 ^c	n.d. ^c	1.01 \pm 0.11 ^{cd}	2.85 \pm 0.33 ^d

n.d., not detected.

Different letters denote significant differences ($P \leq 0.05$) between different samples for the same component.

(5.4 mg/g MW > 10 kDa) and smaller proteins particularly those with MW between 10 and 3 kDa since the ExC contained lower content (1.84 mg/g) than ExA and the fraction with lower MW contained more peptides (1.74 mg/g MW < 3 kDa).

Eritadenine is a water-soluble alkaloid, therefore, it was mainly extracted with cold water when compared with the amount noticed in the mushroom powder (Table 2). The minimal amount that remained in the residues was then almost completely extracted with hot water. These results also suggested that eritadenine was resistant to high temperatures and its extraction yield could be enhanced by increasing the extraction temperature. Eritadenine was 2.2-folds more concentrated in the ExA than in the shiitake powder (Figure 3b), amounts that were only slightly lower than those described in other studies obtained with more complex extraction procedures.^{25,58}

Lenthionine was detected in shiitake powder at similar levels than previously reported.^{13,26} It was also noticed in higher concentrations in the ExA extracts (Figure 3b). Afterwards, only traces remained in ReA but probably the slight increase of temperature together with the high pressure utilized for SFE extraction was sufficient to degrade it being undetected in the following extracted fractions. Lenthionine levels were drastically reduced when extraction temperature was higher than 80°C.^{59,60} Thus, apparently pressurized extractions made the compound more susceptible to degradation.

Ergosterol was mainly extracted with SFE (Table 2) obtaining a highly concentrated ExB extract (88.7 mg/g) (Figure 3c). This result was not surprising since previous works indicated that this lipophilic constituent that can be easily extracted with supercritical CO₂.^{21,61,62} Ergosterol in ExB represented 72% of total sterols since the extract also included other derivatives such as ergosta-7,22-dienol (18.5 mg/g), fungisterol (15.3 mg/g) and ergosta-5,7-dienol (1.5 mg/g). The supercritical extraction was carried out using steel spheres instead of sand as carrier material to facilitate the subsequent ReB separation for further processing, however, ergosterol yields were only slightly lower than usually obtained in similar extractions using sea sand (8.9 and 11.8%, respectively).

Total phenolic compounds were mostly found in the water extracts. Obtained fractions (ExA and ExC) contained, respectively, 3.0- and 2.5-fold more phenols than initially detected in the mushroom powder. The second water extraction complemented the first one leaving in the last residue a very low amount of these compounds (Figure 3c). These results were in concordance with previous studies reporting that water achieved higher recoveries of total phenolic

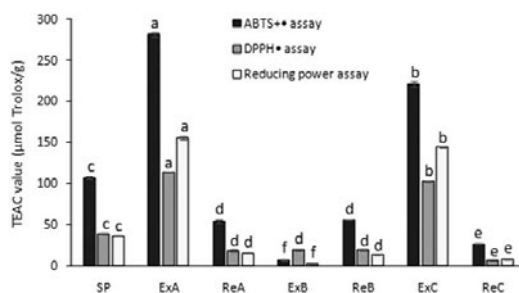


Figure 4. ABTS^{•+} and DPPH[•] scavenging capacity and ferric ion reducing power of shiitake powder and the fractions obtained after the sequential extraction method expressed as their TEAC value.

Different letters (a–f) showed statistical significance ($P \leq 0.05$) between different samples.

compounds in comparison with organic solvents such as methanol.¹⁶

Biological activities of the obtained fractions

The antioxidant activity of the obtained fractions was evaluated as their ABTS^{•+} and DPPH[•] scavenging capacities and as their ferric ion reducing power. Results indicated that those fractions containing high concentrations of proteins, eritadenine and phenolic compounds showed high antioxidant activities (Figure 4). Their levels were more than double of the initial mushroom powder. ExA and ExC were the fractions with the lowest IC₅₀ values (respectively, 0.05 and 0.06 mg/mL when using ABTS^{•+} and 0.15 and 0.17 mg/mL when using DPPH[•]) and the lowest EC₅₀ for their reducing power (0.26 and 0.28 mg/mL). According to their TEAC values, the obtained extracts and residues showed higher affinity for the ABTS^{•+} radical than for DPPH[•]. The use of different solvents might also influence the obtained results since less polar compounds were extracted when methanol was used to carry out the DPPH[•] assay. The shiitake and ExA antioxidant activities were in concordance with previous studies^{16,63} where it was indicated that their antioxidant activities as radical scavengers were mainly due to the phenolic content and ergothioneine.¹⁴ Similarly, the antioxidant activities noticed in the extracts also showed high correlations with their phenolic concentration (with $R^2 = 0.99$ for ABTS^{•+} and reducing power assays and 0.97 for DPPH[•] assays).

Cholesterol lowering in serum can be achieved via several mechanisms but mainly by impairing of exogenous cholesterol absorption or inhibiting endogenous cholesterol

biosynthesis. The hypocholesterolemic activity of the obtained fractions was evaluated as their ability to inhibit the key enzyme of the cholesterol biosynthetic pathway (HMGCR) since the capacity to impair cholesterol absorption was already evaluated elsewhere for similar extracts containing high ergosterol levels (as ExB) and high β -glucans and dietary fibers contents (as respectively, ExC and ReC).^{7,17} The initial shiitake powder showed a remarkable HMGCR inhibitory activity acting as pravastatin used as control (Figure 5). ExA showed similar inhibitory activity (98%) than the mushroom indicating that the responsible compounds might be water soluble. However, the inhibitors were not completely separated with a single cold water extraction since ReA and ReB were still able to lower HMGCR activity (respectively, 86 and 80%). Perhaps, to further improve the extraction yield of HMGCR inhibitors serial extractions with cold water could be carried out although this might involve dilution of the other bioactive compounds. Increase of the water temperature for extraction is not encouraged because after heat application, the inhibitory activity of the obtained fractions was drastically reduced to 31 and 24% for, respectively, ExC and ReC, suggesting that the compounds responsible for the inhibition were thermolabile. This indication was also noticed in previous studies.³³ The ExB extract was also able to inhibit 59% of the enzyme activity indicating that perhaps other compounds soluble in supercritical CO₂ (more lipophilic molecules) were also interfering with the HMGCR activity as was also previously noticed by Gil-Ramirez et al. (2013)⁶⁴ for SFE extracts obtained from *Agaricus bisporus*.

The hypotensive activity of the obtained fractions was evaluated as their capacity to inhibit the angiotensin converting enzyme. However, since it was previously reported that certain peptides were responsible compounds a more detailed

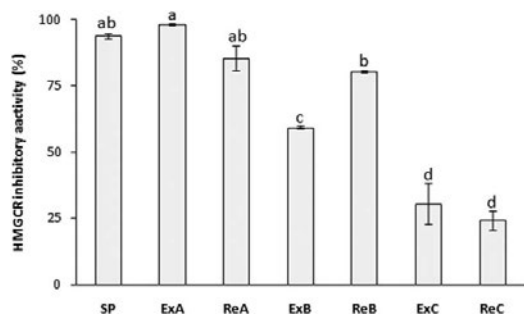


Figure 5. HMGCR inhibitory activity of shiitake powder and the fractions obtained after the sequential extraction method.

Different letters (a–d) showed statistical significance ($P \leq 0.05$) between different samples.

study was carried out on the two extracts including larger amounts of proteins (ExA and ExC) by dividing them in 3 fractions of different molecular weights. The protein contents of fractions obtained from ExA were higher than those from ExC and within the fractions, lower molecular weight fractions contained less proteins, peptides and other N-containing compounds (Table 3). Fractions <10 kDa showed higher IC₅₀ than higher MW fractions ranging from 0.45 mg/mL for the MW > 10 kDa fraction from ExA up to 1.63 mg/mL for the MW < 3 kDa fraction from ExC. These values were lower than those reported in similar studies where other mushrooms such as *Hypsizygus marmoreus* (6.4 mg/mL)¹¹ or mycelia extracts from *Lactarius camphorates* (1.6 mg/mL)¹² were analyzed. A significant correlation between protein content and ACE inhibitory activity was found, however, when IC₅₀ values were expressed taking into account the amount of proteins detected, fractions with lower molecular weight displayed the largest inhibitory capacity. Therefore, these results were in concordance with previous studies carried out on close related species such as *Lentinula polychrous* where small peptides were pointed as responsible compounds of their hypotensive properties.⁶⁵

Conclusions

This study showed that bioactive compounds can be differentially extracted from *Lentinula edodes* fruiting bodies or by-products using a sequential extraction method. Extracts obtained with cold water (ExA) contained high levels of water soluble β -glucans, chitooligosaccharides and other carbohydrates that were pointed as potential responsible for the high HMGCR inhibitory activity showed in this extract. It also contained eritadenine with cholesterol lowering properties, lenthionine that can inhibit platelet aggregation, peptides with ACE inhibitory capacity and antioxidant phenols. Then, the residue after this extraction can be submitted to supercritical CO₂ extraction to obtain a fungal sterol-enriched fraction (ExB) with the ability of displacing cholesterol from dietary mixed micelles formed after digestion impairing its absorption. Afterwards, the remaining material can be submitted to hot water extraction yielding an extract (ExC) containing β -glucans with bile acid-binding capacities (able to interfere cholesterol absorption) and similar solubility than lentinan and a residue (ReC) including dietary fibres such as β -glucans and chitins that apparently, according to previous studies, modulate human microbiota reducing the risk of CVDs. Therefore, many biologically active compounds can be separated within different fractions from the same batch of shiitake mushrooms if the described methods are sequentially applied. Then, the obtained fractions will contain higher concentrations of the bioactive compounds than the mushroom itself and they will be transformed into more bioaccessible forms (i.e., dietary fibers will be more

Table 3. Total Protein Content (PR) and ACE Inhibitory Activity (IC₅₀) of the Different Molecular Weight Fractions Obtained from ExA and ExC

Extract	Fraction	PR (mg/g fraction)	IC ₅₀ (mg fraction/mL)	IC ₅₀ /PR (mg protein/mL)
ExA	MW > 10 kDa	391.93 ± 2.28 ^a	0.45 ± 0.01 ^c	0.18 ± 0.03 ^{ab}
	10 > MW > 3 kDa	237.97 ± 3.34 ^b	0.91 ± 0.08 ^{bc}	0.22 ± 0.02 ^a
	MW < 3 kDa	18.10 ± 1.28 ^f	1.06 ± 0.09 ^{bc}	0.02 ± 0.00 ^c
ExC	MW > 10 kDa	192.81 ± 3.28 ^c	0.74 ± 0.07 ^c	0.14 ± 0.00 ^b
	10 > MW > 3 kDa	87.77 ± 2.28 ^d	1.21 ± 0.09 ^b	0.11 ± 0.01 ^c
	MW < 3 kDa	35.49 ± 1.09 ^e	1.63 ± 0.15 ^a	0.06 ± 0.00 ^c

^{a–f} Different letters denote significant differences ($P \leq 0.05$) between different samples for the same column.

easily fermented by colonic microbiota activating hypocholesterolemic mechanisms or absorbed by M cells in the intestine).

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Conflict of Interest

None.

Notation

ACE = Angiotensin-I Converting Enzyme

CVD = Cardiovascular disease

HMGCR = 3-hydroxy-3-methylglutaryl coenzyme A reductase

SFE = Supercritical fluid extraction

TEAC = Trolox equivalent antioxidant capacity

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